ACKNOWLEDGEMENT

My PhD experience at the University of Nottingham was a period of continuous professional and personal development. This was achieved with continuous support by my supervisor Professor Christine E. R. Dodd over the past few years. Her contribution to my PhD study and to my development is very much appreciated.

I am very grateful of Chris's generous time, support and technical guidance that have enabled me to continuously develop as a researcher and professionally.

I thank Dairy Crest LTD for sponsoring my PhD and for providing the analytical samples used in this study. The analytical and basic microbiology works in the study were done at the Dairy Crest Laboratory.

I thank Mr Mark Litherland, Technical Services Manager at Dairy Crest for his support since 2009 to carry out the research wok and the cooperation of Mr Kerry Croxton, Analytical Manager is also thankfully acknowledged.

I would like to thank all the staff and colleagues at the Division of Food Sciences and particularly Wendy Fielder and Lorraine Gillett and fellow student Dewi Yunita for their day to day help with my laboratory work.

Finally, I believe that everything in life is a matter of choices and priorities and in order to complete this study I had to sacrifice further advancement in my career and personal life. I am grateful that I found the courage to do so throughout my PhD years.

Finally, I thank my husband Irfan for being so understanding during this period looking after my young son and doing all the household chores while I was busy with my samples.

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ABSTRACT

The overall aim of this study was to eliminate the root cause of the 'cheese flavour' in spread caused by four key microbes Bacillus spp, Staphylococcus spp, yeasts and moulds. The major sources of these bacteria were in the product ingredients mainly sweet cream buttermilk and skimmed milk along with environmental aerosols. The causative organisms were present in about 63% of the products and mainly 'feed' on the oil element of the recipe, containing high level C12 that generates the distinctive cheese flavour when broken down by bacteria.

The key hurdle factor in spread preventing microbial growth is water droplet size. The spread showing cheese off flavour had a droplet size distribution of 95% <10 micron. To achieve finer droplet size distribution, trial products were made in the Scrape Surface Heat Exchanger (SSHE) over the current churning method with a distribution of droplet size 98% <5 micron. The trial product showed a 50% reduction in the generation of the 'cheese flavour' methyl ketones. The Staphylococcus spp cross contamination source where from personnel with direct food contact processing area. Further education on personal hygiene helped to reduce the level of Staphylococcus spp contamination in the product. The trial product from the SSHE was further challenge tested with Listeria monocytogenes over a 10 week shelf life period to evaluate product robustness against microbial growth and spoilage. The organism did not show any growth over the period of time. The liquid phase of the emulsion was further modified with various salts at

different concentrations and challenged with L. monocytogenes isolated from various parts of the dairy environment. It was observed that a pH range of 5.5 or lower with added 0.063% potassium sorbate showed significant antibacterial affect compared to the nutrient enriched MPC-broth and the unsalted liquid phase of the emulsion with no added potassium sorbate.

To understand L. monocytogenes survival within a dairy process, the organism was further challenged by exposure to pasteurisation heat treatments and the standard CIP cycle of acid and caustic treatment. No recovery rate of the organism was observed.

Therefore it could be concluded that the contamination within the industry is more likely to be post process or environmental contamination rather than survival through the plant itself as per RASFF alert of Listeria spp outbreak in dairy. Therefore, reducing the available water in the liquid phase of the spread and achieving a <5 μm droplet size and a finer distribution within the product will be limiting factors to microbial growth.

An air purifier system BAXX has reduced the level of environmental contaminants, especially yeast and mould.

CHAPTER 1 INTRODUCTION

Milk, cream, butter, margarine and dairy spreads exist as emulsions at different stages of their production (Dickinson 1989; Friberg and Yang 1998, McKenna 2003). The overall quality of an emulsion-based product is a function of a combination of physiochemical and sensory characteristics that contributes to the product specific characteristics. These can determine the rheological characteristics with respect to viscosity, appearance, aroma, taste, shelf life and texture. The raw materials, such as oil, emulsifiers, thickening agent, minerals, acids, vitamins, and flavours and water, along with the specific processing conditions (mixing, homogenization, pasteurisation etc.), determine the product quality attribute, chemistry, sensory and shelf life (McKenna 2003).

A spread emulsion is made up of two immiscible liquids, such as oil and water, where one of them is dispersed in small droplets in the other (Rajah 2002). The diameter of the droplets in the spread emulsion can vary from 0.1 to 100 µm.

An oil -in- water emulsion is where oil droplets are dispersed within water. For example milk and cream are oil in-water emulsions. The product structure is affected by melting, solidification, whipping, viscosity, de-emulsification and stability. On the other hand, water droplets dispersed in oil make a water-in-oil emulsion such as in margarines, spreads and butter, which is similarly affected by the melting and solidification, stability, plasticity, consistency, spreadability and viscosity (Rajah 2002, McKenna 2003).

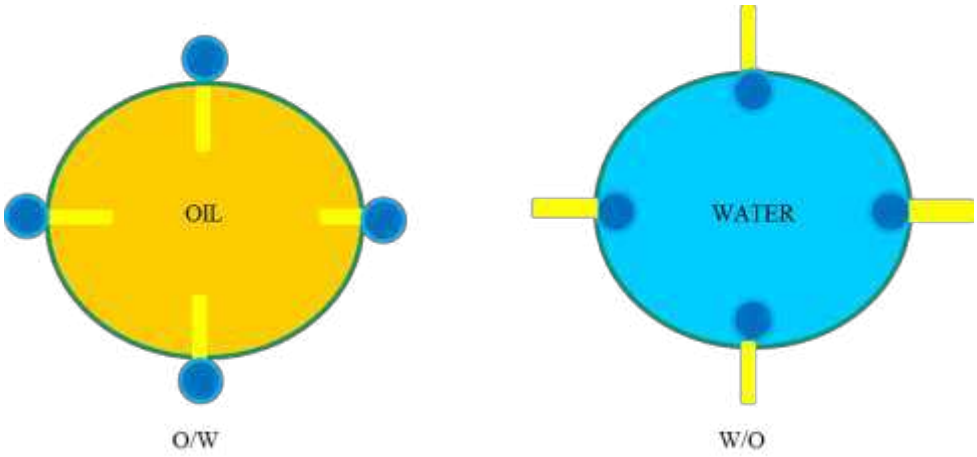


Figure 1.1 An oil-in water (left) and water-in-oil (right) emulsion (Adapted from McClements 1999)

An emulsifier consists of a water-loving hydrophilic head and an oil-loving hydrophobic tail. The hydrophilic head is directed to the aqueous phase and the hydrophobic tail to the oil phase (Figure 1.1). The emulsifier positions itself at the oil/water or air/water interface and, by reducing the surface tension, has a stabilising effect on the emulsion.

Milk proteins derived while separating milk from cream during processing such as skim milk or other process by-products as whey powder or buttermilk have an oil / water emulsion compared to spreads where the structure is a water /oil emulsion. This type of emulsion destabilises the product structure but works as a flavour enhancer compared to oil / water emulsions.

1.1 SPREADS

Spreads includes any plastic fat composition emulsified to at least 80% fat and with moisture in excess of 1% (EC 1994; ICMSF 2005). A wide range of fats and oils can be used to make a spread via various processes. Other ingredients include salt, emulsifiers and preservatives, and some spreads may contain milk solids. Others may contain 40% to 60% fat with a corresponding increase in moisture content. Spreads have various oil contents and usually do contain milk solids.

A wide range of fats may be used (e.g. palm, sunflower, soya oils). In addition to the fat-blend, water is required at 15-20% of product weight; emulsifiers are also required. Some countries, including the UK, prefer margarine with high salt levels, but for dietary reasons salt may be completely omitted. The concentration of total salt may vary from 0 to 2% w/w in the finished product, which is equivalent to 0-11% w/w in the aqueous phase. Milk or milk products are generally added in powdered form in concentrations of 0.1-1.0% of the product. Vitamins A and D, together with flavourings and colourings such as beta-carotene can also be added for a nutritional claim. Adding vitamins to margarine was a legal requirement though this no longer exists (FSA 2010). Voluntary fortification of margarine with vitamins has been practiced by manufacturers since 1925, but in 1940 with the advent of the Second World War, certain Governments took action to safeguard the nutritional status of the nation by making the addition of vitamin A and D compulsory. This mandatory fortification was justified in the view that margarine was being used to replace butter in the diet. Organic acids

e.g. lactic acid, are added to spreads as acidity regulators. The USA also permits antimicrobial preservatives such as sorbic acid at a maximum concentration of 0.1% of the product, and benzoic acid at 0.1%, or a combination of the two. In Western Europe, only sorbic acid or its salts are allowed, with a maximum acid concentration of 0.1% of the product (EC, 1995; ICMSF, 2005).

"Fat Spread" is a water-in-oil emulsion but with vegetable oil predominating and only a small amount of cream present with added emulsifiers. "Blended Spread" is a water-in-oil emulsion with approximately equal amounts of butter and vegetable fats.

Products with a fat content of between 41% and 62% are commonly referred to as reduced fat spreads (ICMSF 2005). There are several differences between an 80% fat and 40% fat water-in-oil emulsion. Firstly the moisture content of the reduced fat product is higher and salt and water and other water-soluble preservatives are diluted. The dilution factor cannot be compensated by the addition of more agents due to unacceptable organoleptic results. In order to structure the aqueous phase of the reduced fat product, there is a tendency to add biopolymers (vegetable or protein, thickeners). The water droplet size has a higher mean diameter and a larger distribution width compared to margarine products because of physical effects (Rajah 2002).

The shelf life of closed (i.e. unopened) packs of reduced-fat spreads is generally limited to 3-6 months, often not for microbiological reasons but for sensory ones.

Edible spreads include milk fat spreads, margarine fat spreads and blended spreads as per Table 1.1 (Codex 1999). The difference is based on the percentage of fat. The difference in formulation determines the end product appearance, texture, taste, spreadability and shelf life (Charteris 1995), the major ingredients being the oil, milk, protein and additives.

Table 1.1 Categories of Table Spread (Codex 1999)

Product Group	**Composition**	**Type**	**% Fat**
Butters and Spreads	100% Milk Fat	Butter Spread Reduced Fat Butter / Spread Low Fat Butter / Spread	80-95 >61-80 >59-41 ≤39
	3% Milk Fat (Minimum)	Margarine Spread Reduced Fat Margarine / Spread	80-95 >61-80 >59-41
	15-80% Milk Fat	Blend Blended Spread Reduced Fat Blended Spread Low fat Blended Spread	80-95 >59-80 >39-59 ≤39

1.1.1 Market size and trends

DEFRA (2006) in their national statistics mentioned that since 1974 consumers have been replacing butter, margarine and other fats by low fat butters, reduced fat butters and vegetable and salad oils (Table 1.2) (‘'Family Food in 2003-04') with butter purchases having falling by 41%. There is a continuing move away from ‘reduced and low fat spreads’ which fell by 16% since 2006. Purchases of butter dropped 3.6% although the price rises that had been seen in 2008 were slightly reversed. The trend in purchase expressed as grams per person per weeks of fat (adapted from Family Foods 2013, DEFRA 2014) is shown in Figure 1.2, which shows a general trend of fat purchase since 1974, with a continuing move away from butter and margarine and increasing trend of consuming low and reduced fat spreads.

Table 1.2 UK household purchases of fats and oils 'Family Food in 2009' (DEFRA National Statistics 2010)

Fat & Oil based products	Grams per person per week				% Change since	
	2006	2007	2008	2009	2008	2009
1.Total fats and oils	184	181	184	181	-1.8	-1.6
1.1.Butter	40	41	40	39	-3.6	-2.7
1.2.Margarine	18	19	22	24	+9.9	+33.4
1.3.Low fat and reduced fat spreads	57	53	51	48	-6.2	-16.0
1.4 Other fats and oils	69	68	72	71	-1.2	+1.9

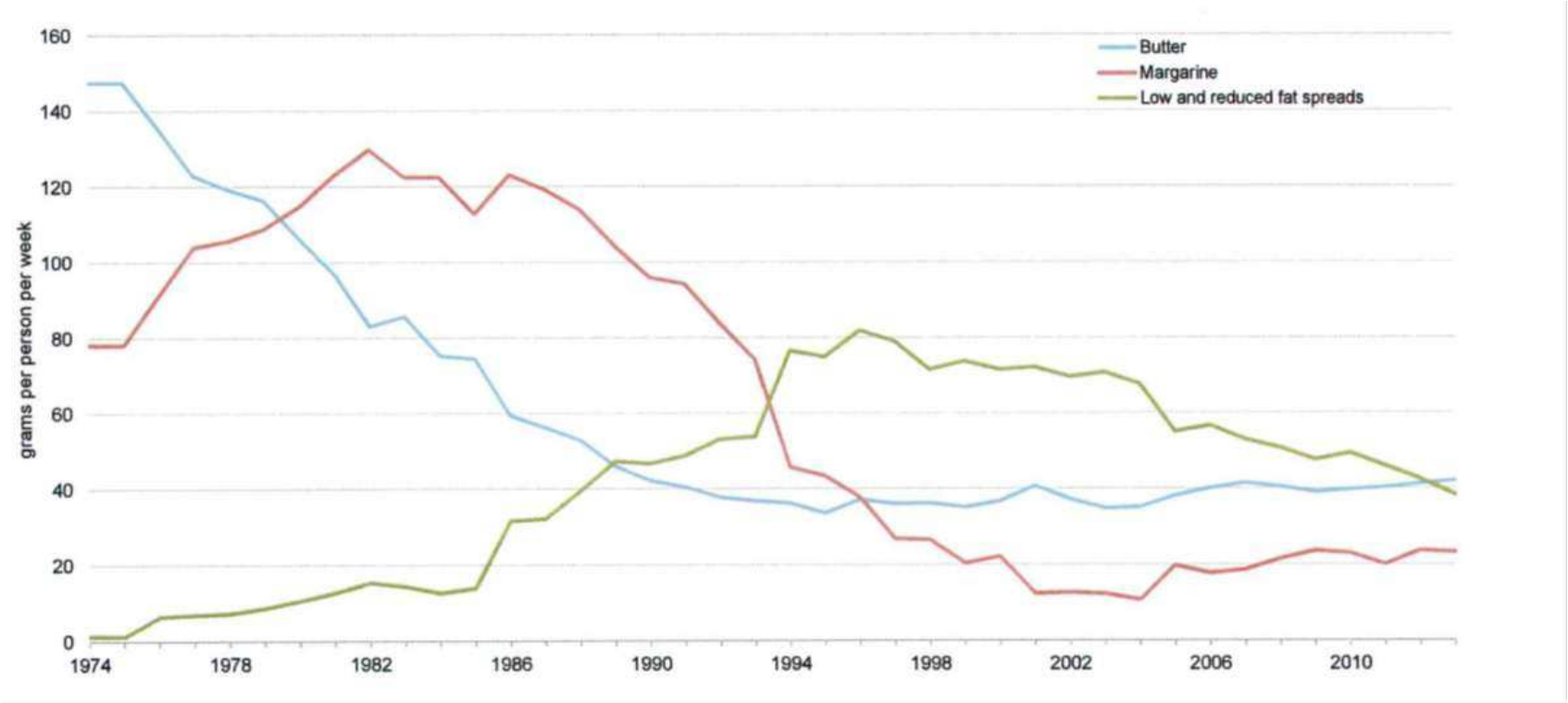


Figure 1.2 Purchase of grams per person per weeks of fat (adapted from Family Foods 2013, DEFRA 2014)

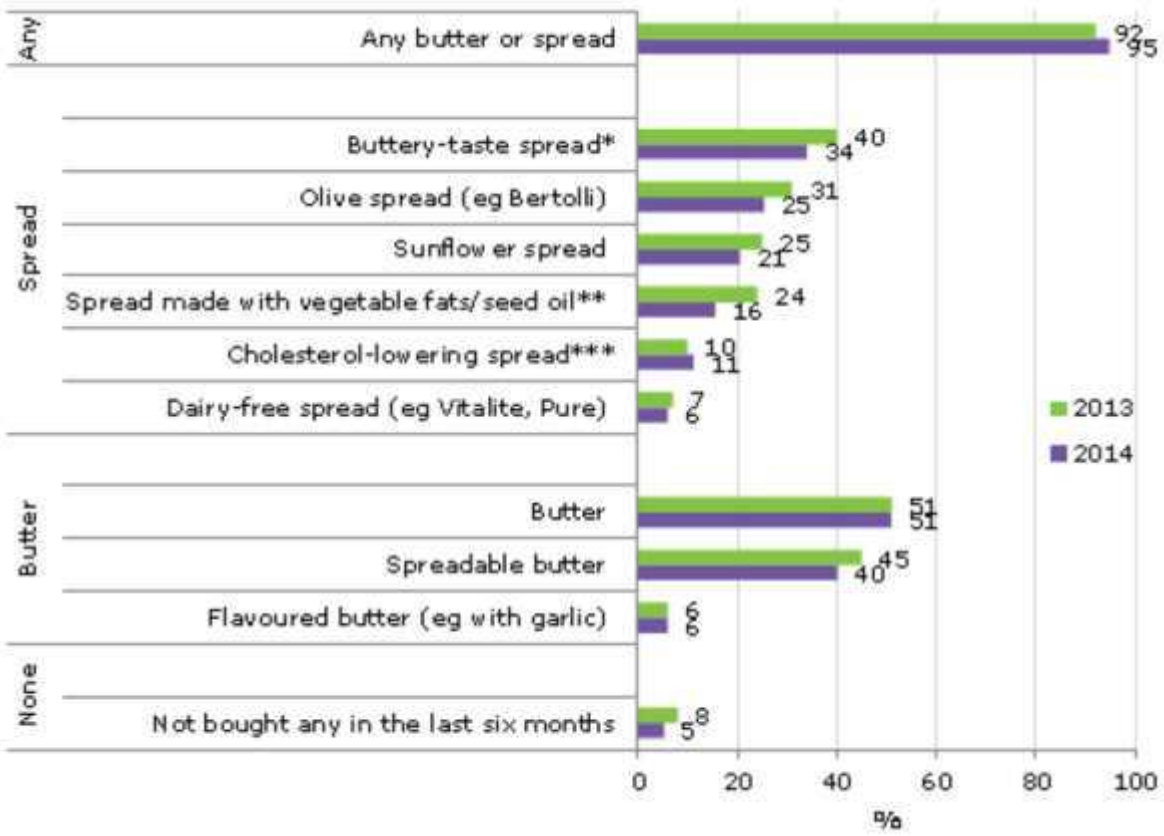


Figure 1.3 Types of yellow fat bought in 2013 and 2014 (Mintel August 2014)

Figure 1.3 (adapted from Mintel 2014 report) shows a survey of the type of yellow fat usage based on 2000 internet users aged 16+. Buttery taste spreads were bought most 40% in 2013 and 34% in 2014 to the total market of 92% in 2013 and 95% 2014 which butter was of 50% of the total volume in 2013. This is probably related to the taste of the product.

A survey (Mintel 2003) of 25,000 UK adults reported that butter and spread showed a similar level of household usage to each other. Low-fat spreads are used by over half of adults where a heavy consumer will consume the product more than once a day, medium user once a day and light user being two or three times a week or less.

Table 1.3 summarises the total yellow fat and spreads value summary and Table 1.4 illustrates the volume summary from the Mintel report in August 2014. As per Table 1.3 all butter sectors were in growth in 2014 while spreads declined being driven by Wellbeing, but dairy spreads was declining at a slower rate than the total. A similar pattern is observed in Table 1.4 where sectors are in volume and spreads were down in volume sales. Table 1.5 summarises the total market snap shot of spreads (Mintel 2014) showing the volume sales side. Private Label has over taken Lurpak as the number one supplier in the yellow fat and spreads market. Country Life, President and ICBINB are also growing their Volume in the year.

Table 1.3 Total yellow fat and spreads value summary (Mintel August 2014)

52 week is for the financial year 2012-2013

12 week for the first 12 weeks of the financial year 2013 - 2014

Table 1.4 Total butter and spreads volume sales summary for 2012-2013 and first financial quarter in 2013-2014(Mintel August 2014)

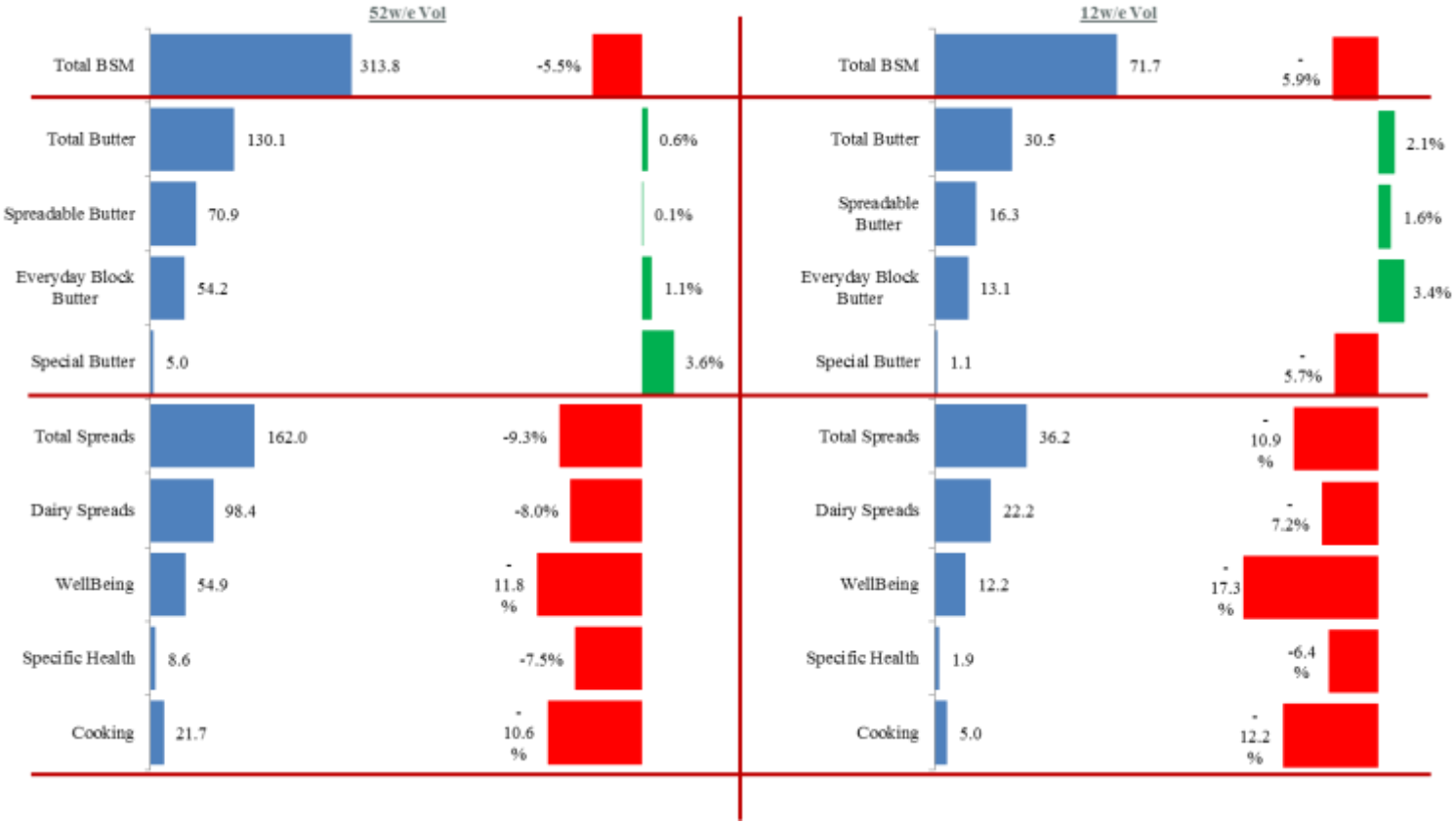
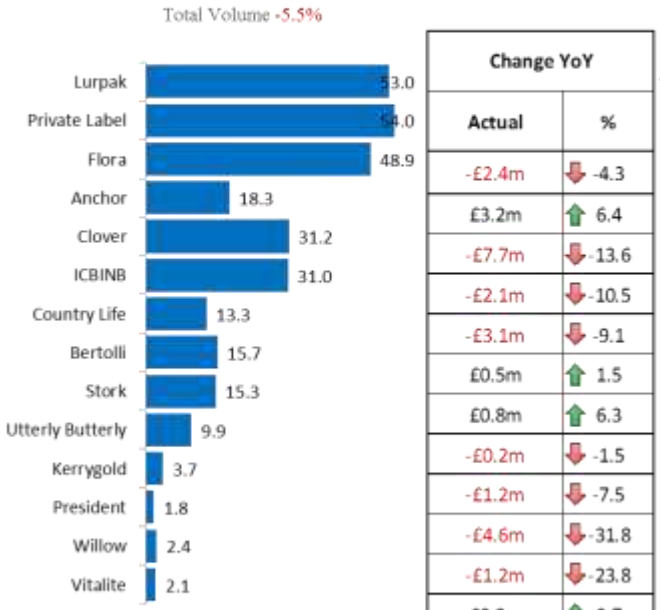
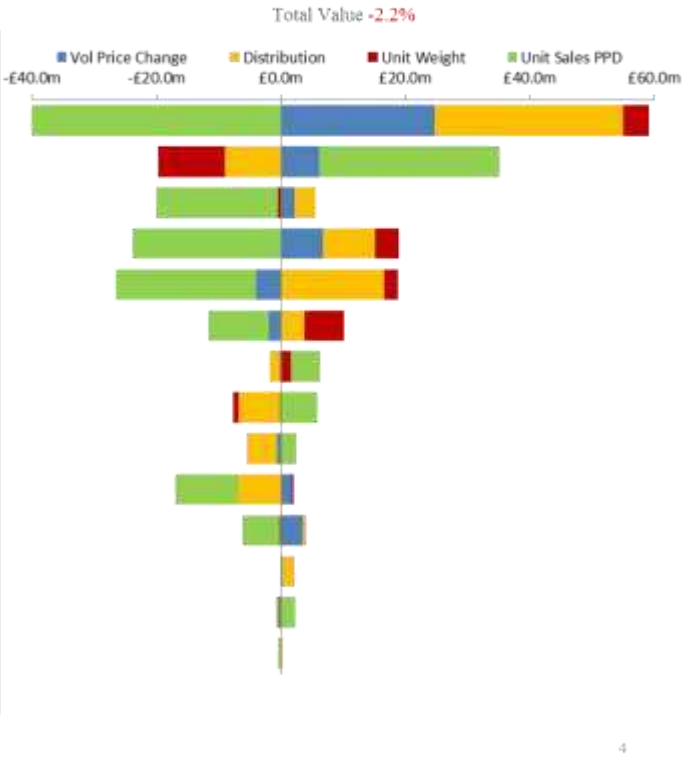


Table 1.5 Total yellow fat and spreads market brands value and volume summary (Mintel August 2014)



Change YoY	
Actual	%
-£2.4m	-4.3
£3.2m	6.4
-£7.7m	-13.6
-£2.1m	-10.5
-£3.1m	-9.1
£0.5m	1.5
£0.8m	6.3
-£0.2m	-1.5
-£1.2m	-7.5
-£4.6m	-31.8
-£1.2m	-23.8
£0.2m	9.7
£0.3m	14.2
-£0.2m	-6.8



1.1.2 Processing

The major spread / margarine development was in 1927 with the addition of vitamins to the spreads and margarines. Spreadable products filled in tubs were introduced to the market in the 1960s (Rajah 2002).

1.1.2.1 History

The early food manufacturers used very basic machinery for manufacturing spreads. Manufacturing steps were mixing, emulsification, chilling, working and resting. Later a mechanical a churn drum was used to mix the oil and the aqueous ingredients for a stable oil in water emulsion (Dickinson 1989; Friberg and Yang 1998, McKenna 2003). A cooling jacket along with ice as an ingredient for the churning was used to facilitate the cooling mechanism. The churns were used to store the mix for fat crystallisation and separation for 24-hours. The wet chilling process was developed into an open dry chilling drum in the 1920's, where the water in oil churned emulsion with added ingredients was passed through a trough and then crystallised in a wagon and further filled into finished tubs. The drawback of all these processes was that they were not fully enclosed for food safety and quality purposes.

An enclosed processing system, the scraped surface system, was developed in the 1940's with a fully enclosed system consisting of the crystalliser, resting tubes; product feed line from the churn to the packing machine. The scrape surface tubular action worked in the following ways (Rajah 2002):

1. Phase Inversion Process where the fat undergoes inversion and is cooled and textured mechanically with cream containing fat at a level of 40%, 60% and 75% of the formulation
2. Ammix Process where the cream is added to the anhydrous milk fat (AMF) to produce a spread with 80% fat.
3. Pasilac Process which uses a mixture of butter and vegetable oils to produce 80% or lower fat spreads.
4. Anhydrous milk fat (AMF) process where cream is added to milk solids to produce a product with 80% or less fat.

The spread could be made by either batch or continuous process (Bullock et al. 1971) (Figure 1.4). The main difference in the process is the tempering method to achieve the optimum consistency of fat and a continuous feed of the liquid phase consisting of water, salt, nutrients and starch, which further determines the droplet size of the product.

1.1.2.2 General Modern Day Spread Processing

Cream is a by-product of a milk processing plant. It is one of the main ingredients in the processing of a spread (Henning 2006). It is a high-risk product in terms of vulnerability to contamination. Prior to processing, the cream is pre-pasteurised at 83ºC (or lower) for 20 seconds (company spread manufacturing parameter) and then stored in an aging tank for a maximum of 48 hours. The cream is then mixed with water, buttermilk, emulsifier powder, oil and vitamins and pasteurised at

83ºC (Figure 1.5). The emulsion is then stored in a silo at 5ºC, passed through a balance tank into a heat exchanger and churned to separate the buttermilk from the spread. Salt is then added to the spread as a slurry. Salt is a microbiologically low risk ingredient and hence a non-safety hazard to the food safety of the spread.

The separated buttermilk, a by-product of the production process, is further recycled and pasteurised at 72ºC for use as an ingredient in the spread processing, although some buttermilk is also dried for sale to customers. The spread then goes through a scraped surface heat exchange for packaging into different sized tubs such as 250 g, 500 g, 1 kg and 2 kg etc.

The rejects due to weight or mislabelling are usually sent off the line to a remelt plant, where product is taken out of the tub and kept in a temperature controlled room at less than 5ºC. Further processing of these items is done at 60ºC. The product is then downgraded and sold separately while being monitored for microbiological and chemical properties. Processing of the lighter variety of spreads is carried out in the same way except that further water and soft oil are heat treated and added according to the recipe for finished spread prior to packaging for distribution.

1.1.2.3 Scraped Surface Heat Exchangers (SSHEs)

Scraped surface heat exchangers (SSHEs) are widely used in the food industry. The process comprises a cylindrical steel annulus and rotating blades to scrape

food away from the temperature controlled outer wall, maintain the mixing and heat transfer (Figure 1.6) (Duffy et al. 2010). The gaps between the scraper blade and walls of the SSHE have a rotating dasher with rows of scraper blades (Figure 1.7). The product is pumped through the cylinder as the heating or cooling medium is circulated around the cylinder. The heating medium can be steam or water as a cooling medium using a refrigerant such as ammonia or Freonn etc. (Harrod 1986) can be used..

The emulsion is chilled and crystallised in the SSHE. While producing spreads of various fat levels, the fat is blended along with all the raw materials in the process. The various stages of spread production are preparation of the water and fat phase, emulsion preparation, pasteurisation, chilling and crystallisation (Stall and Anderson 1986). The major ingredient in the fat phase is fat blended with different fats and oils, with an objective to obtain solid fat content (SFC) at temperatures ranging from 4ºC to 50ºC according to the product type (Stall and Anderson 1986). The fat phase also consists of emulsifier, lecithin, flavour, colour and antioxidants. The water phase consists mainly of water along with salt, sugar, preservatives and milk protein. Salt is used as a flavour enhancer that also prevents the growth of microorganisms whilst citric acid is used to lower the pH which is also used as a preservative. Other preservatives such as benzoates or sorbates can be used to prevent the growth of spoilage microorganisms (Rajah 2002).

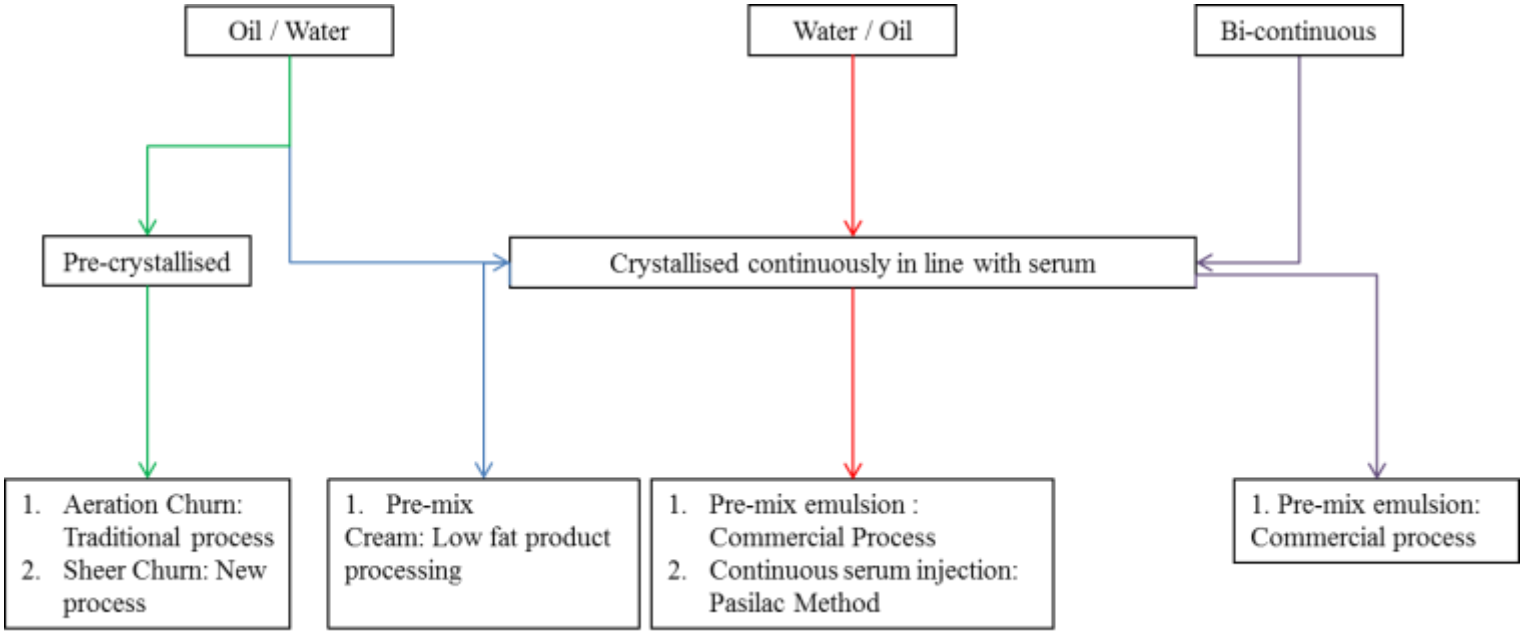


Figure 1.4 Manufacturing Process of Spreads oil / water, water / oil (adapted from Charteris 1995)

1. Churn Process
2. Low Fat Process
3. Water / Oil Pasilac or Batch process
4. Water / Oil Bi-continuous Process

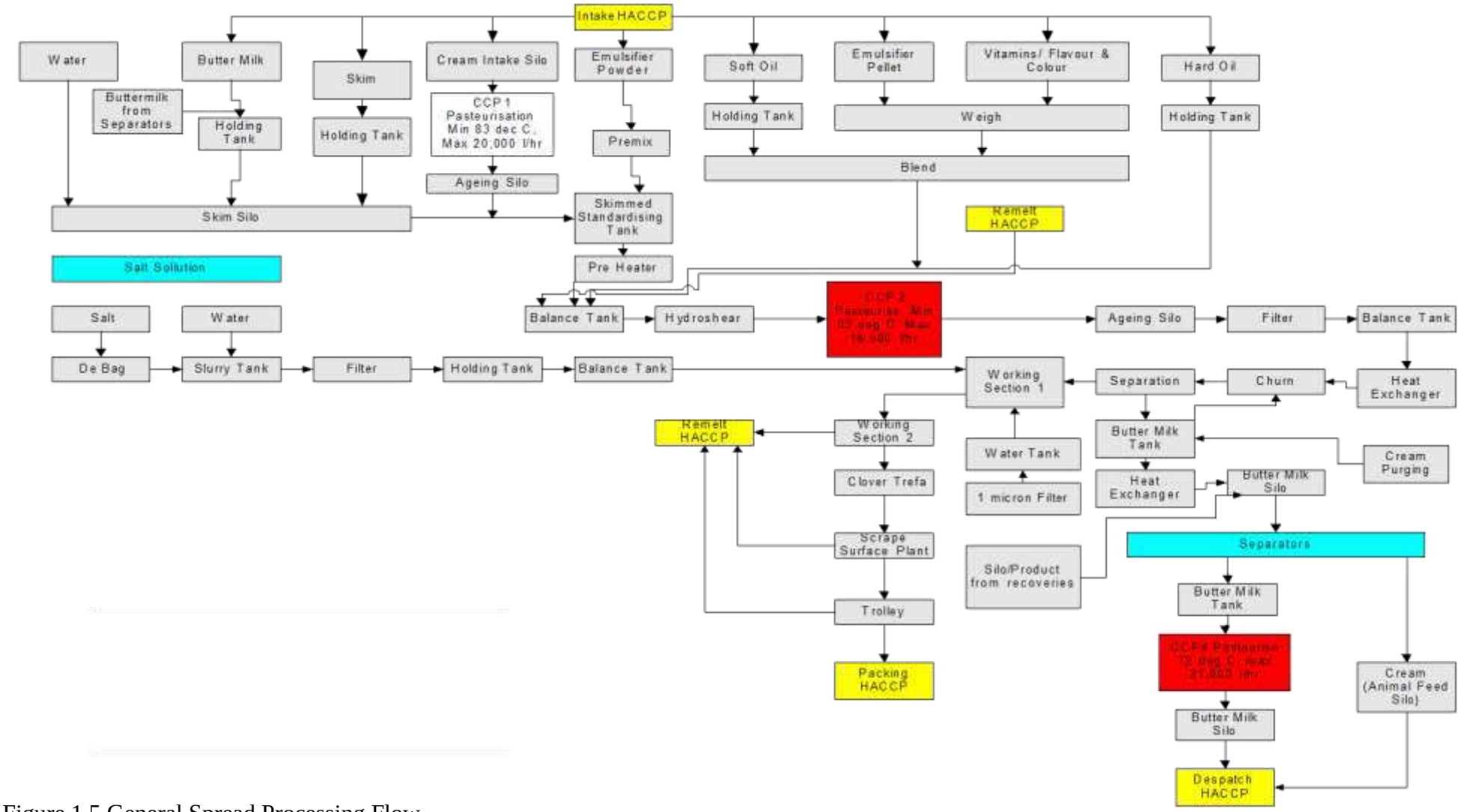


Figure 1.5 General Spread Processing Flow

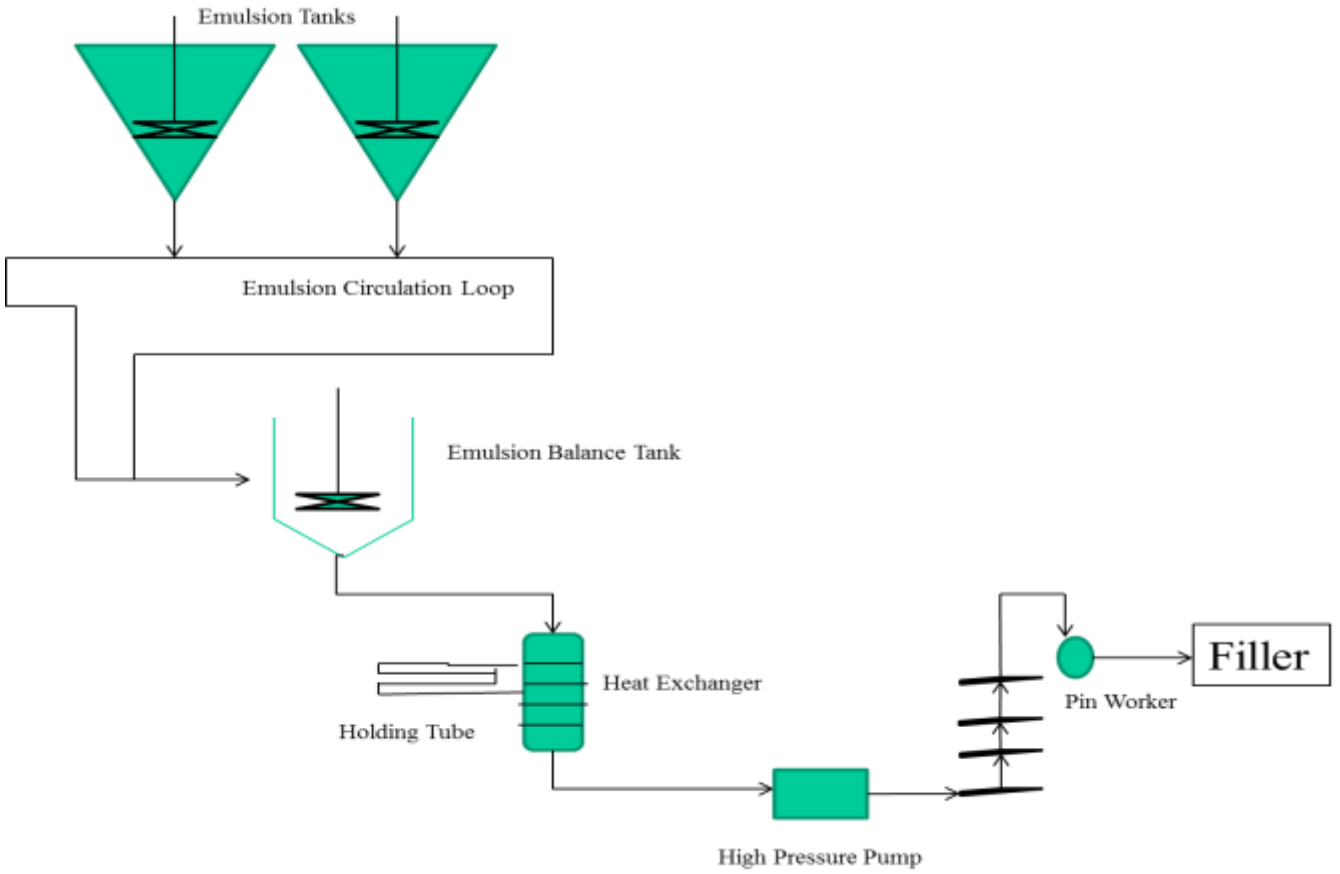


Figure 1.6 Scraped surface heat exchange spread processing

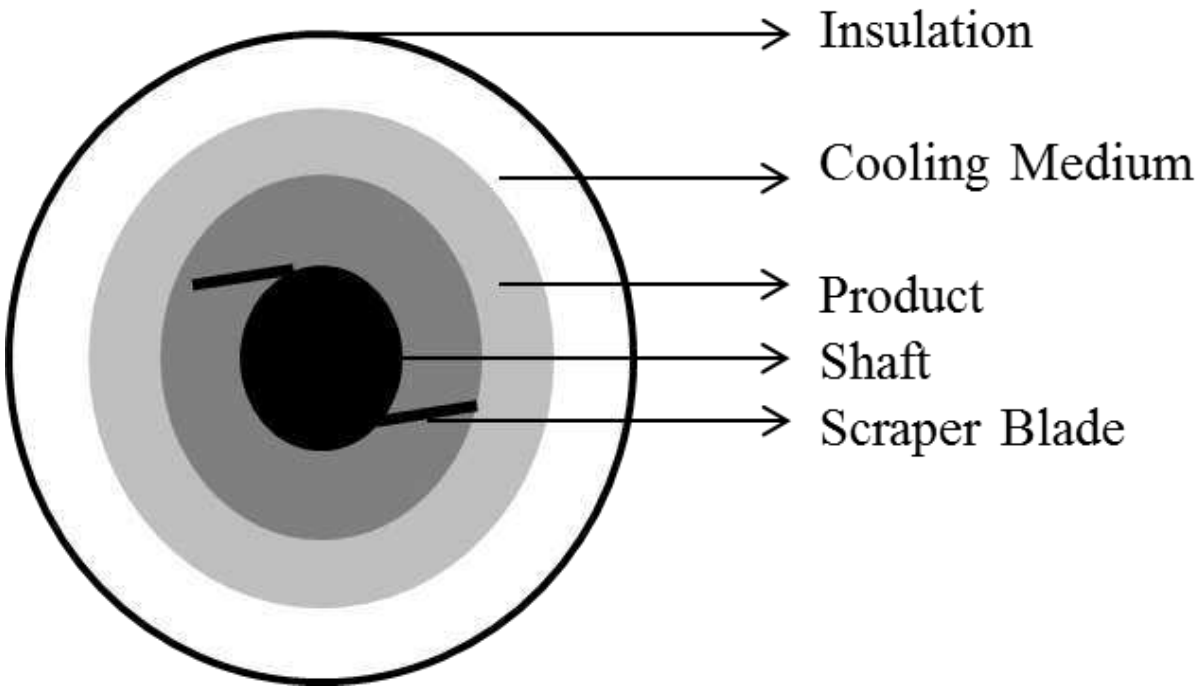


Figure 1.7 Cross Section of SSHE (adopted from Duffy et al. 2010).

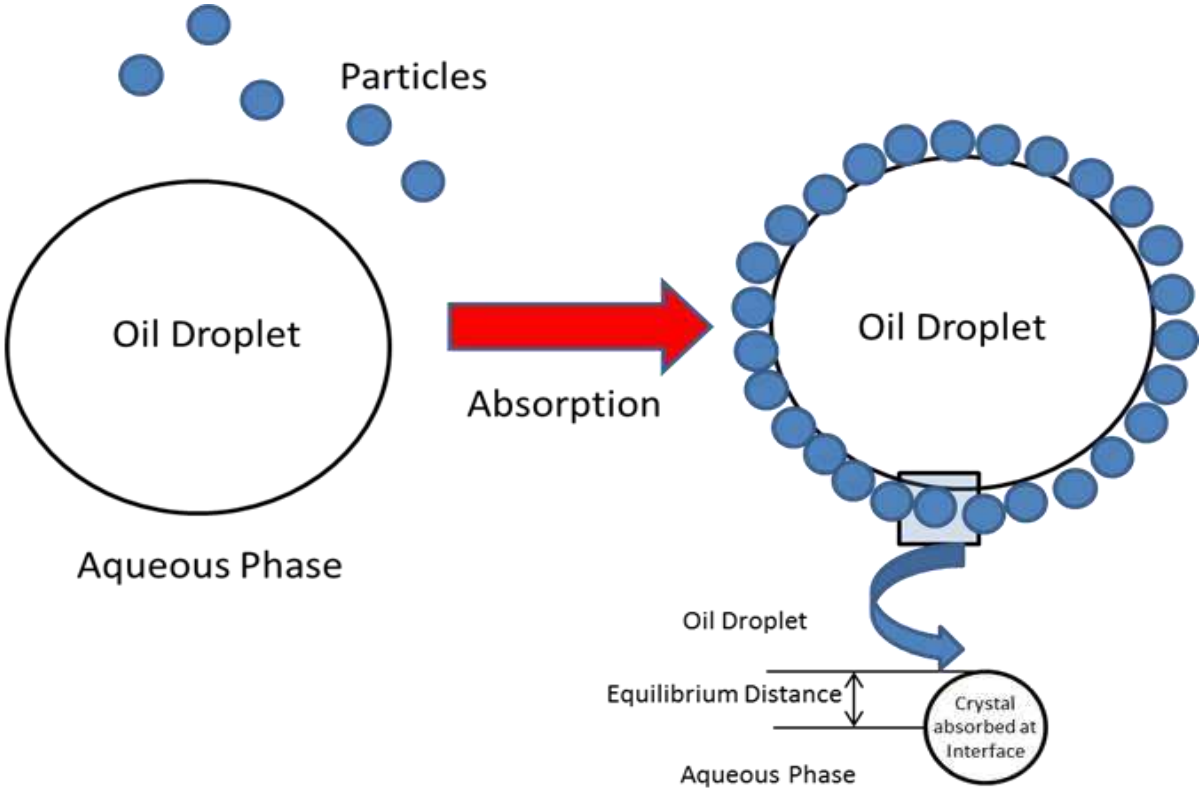


Figure 1.8 Absorption of fat crystals at the interface of an oil-in-water emulsion (adapted from Rousseau 1999).

1.1.3 Formulation

Spread processing, preparation and the choice of ingredients is product dependant. The oil and water phase are prepared separately and heat-treated to stabilise them prior to emulsification. It may also involve single or a number of consecutive steps to disperse the ingredients in the phase where it will be most soluble (McClements 1999). Oil soluble ingredients are usually vitamins (Vitamin A, D, E and K), colours, antioxidants and surfactants whereas proteins, polysaccharides, sugars, salts, vitamins (Vitamin B complex and C) are usually water soluble. The process that involves converting the two immiscible liquids into an emulsion is called homogenisation which is carried out mechanically by a homogeniser. This is usually carried out in two steps, the primary and the secondary homogenisation. The former, involves formation of an emulsion primarily from two separate liquids, whereas the latter will reduce the size of water droplets in the existing emulsion (McClements 1999) which gives rise to smaller droplet size.

Figure 1.9 shows the two - stage mechanism for producing emulsion droplets coated by a two-layer interfacial membrane, firstly, a primary emulsion containing small droplets coated with an emulsifier membrane is formed by homogenizing oil, water and lecithin together. Secondly, a secondary emulsion is formed by mixing the primary emulsion with a chitosan solution to form droplets that are coated with a lecithin-chitosan membrane.

Along with the effect on organoleptic, functional and nutritional properties, the liquid phase forms the fine water droplet molecules in the product structure; it also encompasses the microbiological quality of the final product (Charteris 1995). To prevent the growth of microorganisms in spreads, variation of both the chemical formulations as given in Table 1.6 and the processing parameters along with storage temperature have a vital role in both the oil-in-water and water-in-oil products.

The most common emulsifiers are monoglycerides or a mixture of mono and diglycerides. Saturated monoglycerides help to achieve a stable emulsion. Soya lecithin improves the emulsification by delaying the onset of the oxidation rate (McClements 1999). This then reduces spattering while cooking, along with being a good antioxidant that can improve the shelf life of the products.

Emulsifiers are surface- active compounds used to reduce the interfacial tension between the water and fat phase. They stabilise the liquid emulsion prior to crystallisation for a homogenous product and to achieve finely dispersed water droplets in order to improve the microbial keeping properties of spread (McClements 1999).

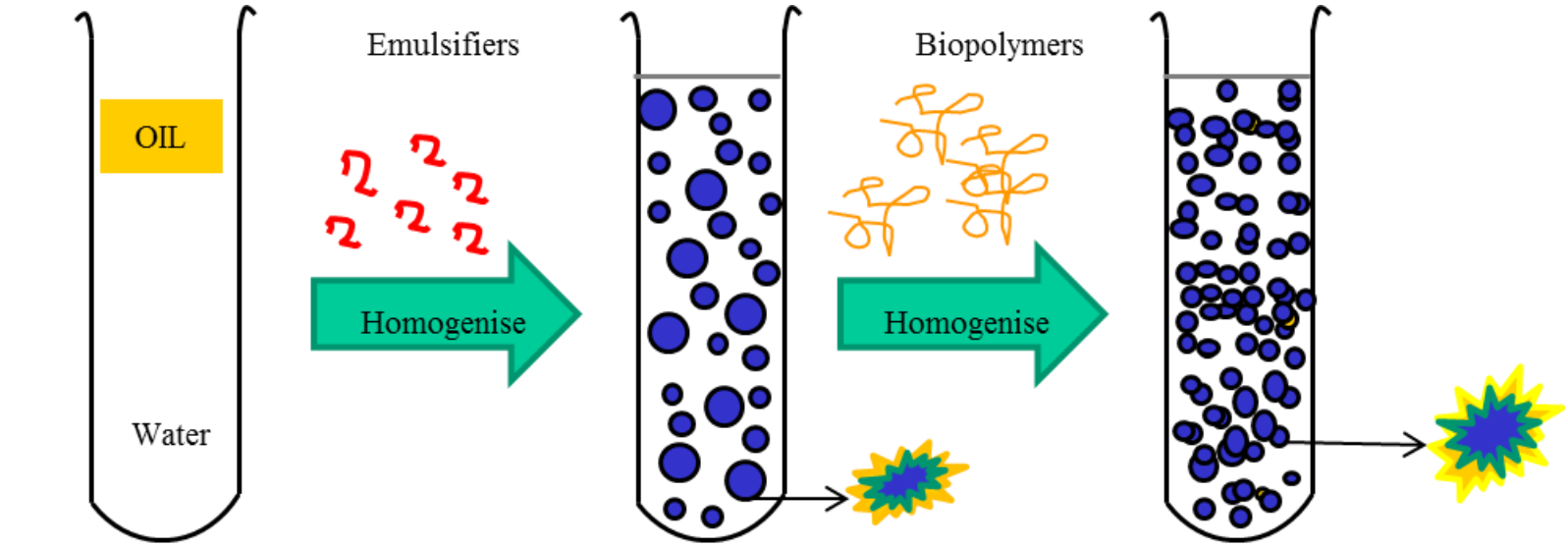


Figure 1.9 Primary and secondary homogenisation of emulsion formation (McClements 1999)

Table 1.6 The principal ingredients as per Codex (CAC 1996) which affect the microbiology of spread

Agents	Examples
Processing Aids: Bacterial Cultures	*L. lactis* spp *lactis*, spp *cremoris*, spp *lactis* biovar *diacetylactis*
Food Additives / Preservatives	Sorbic acid, Sodium/Potassium/Calcium Sorbate, Benzoic acid, Calcium/Sodium/Potassium Benzoate
pH correcting agents	Acetic acid, Lactic acid, Citric acid, Lactates, Sodium/ Calcium hydroxide etc.
Miscellaneous	Argon, Nitrogen, Nitrous oxide, H_2, O_2, CO_2
Others	Emulsifiers, thickeners / stabilisers, antioxidants
Optional	NaCl, egg yolk, edible protein, starches, milk protein, oligosaccharides

1.1.4.1 Proteins

The main source of protein in dairy spreads is milk protein in the form of skim milk, whey, butter milk. It imparts the creamy taste and texture along with making a contribution to the aqueous phase viscosity and stability, thus improving the emulsion stability through the life of the product (Charteris 1995). The proteins are also a good source of nutrients, being a source of essential amino acids. The amino acids on the other hand are a good source of nutrients to microorganisms and facilitate in their rapid growth at temperatures above 10ºC. As per the Food Hygiene Regulation (1996) all chill products should be held at ≤8ºC to restrict the growth of pathogenic microorganisms.

1.1.4.2 Lactic Acid

Lactic acid (E270) and its sodium (E325), potassium (E326) and calcium (E327) salts act as acid regulators in the products. These could be added to the aqueous phase directly but will give a lactic / acidic taste. Hence they work both as flavour enhancers and as an antimicrobial agent (Voysey 2009).

Both dissociated and undissociated forms of lactic acid exist in equilibrium in the water phase and the undissociated ionic form is the antimicrobial form (Charteris 1995).

1.1.4.3 Preservatives

As per CAC (1996) the preservatives allowed in butter and spreads are Sorbic acid (E200) and Benzoic acid (E210) along with sodium, potassium and calcium salts. The maximum permissible levels are 0.1% w/w. When used in combination Sorbic acid (E200) and Benzoic acid (E210) should not exceed 0.2% and 0.1% respectively.

They are both used as food preservatives along to provide a broad spectrum antimicrobial activity especially for spoilage microorganisms such as yeasts and moulds (Charteris 1995). The antimicrobial activity of sorbic acid and potassium sorbate is effective up to pH 6.5 but the antimicrobial activity decreases with lower pH. On the other hand benzoic acid is effective at a pH range of 2.5 to 4.0.

The ability of a microorganism to grow in an emulsion is determined partly by the pH of the aqueous phase. The pH in margarine is usually between pH 3.5 and 6.0. Citric acid and lactic acid are commonly used as acidulants in spreads (Holliday et al.2003). The effects of pH and salt levels on the growth of L. monocytogenes, concluded that combinations of pH (3.8-5.5), and total salt levels between 1.5- 5% prevented the growth of L. monocytogenes at 5, 10 and 23ºC. The pH of cultured butter is usually between 4.6 and 5.3 and sweet cream butter typically has a pH of 6.6.

Rosemary and several of its constituents, including carnosic acid and carnosol, have exhibited antibacterial effects against various Gram-positive and Gram-negative bacteria in vitro including oral planktonic bacteria Bacillus subtilis, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, methicillin-resistant Staphylococcus aureus (MRSA), lactobacilli, Listeria monocytogenes, Streptococcus thermophilus, Pseudomonas fluorescens, and Yersinia enterocolitica (Abramovic et.al. 2012).

1.1.4.4 Sodium Chloride

Sodium chloride addition works as a flavour enhancer as well as having an antimicrobial activity. The antimicrobial effectiveness is dependent on its effect on the availability of water or water activity. For both oil-in-water and water-in-oil emulsions, it is very difficult to measure their a_w value (Gomez and Fernandez-

Salguero, 1992). The aqueous phase is separated from the oil phase where this is needed, and the water activity of the aqueous phase was measured.

For spreads containing higher water contents, microbiological safety becomes a concern. The aqueous phase is pasteurised to minimize the number of microorganisms. The survival of a microorganism in a nutrient-poor emulsion is determined by its 'stress tolerance response' (Dodd 2005). The adaptation of the microorganisms to allow them to grow outside the range of their tolerance is due to the stress response. The cells have greater tolerance to stress during the stationary growth phase rather than exponential growth phase. Global stress response regulon sigma factor helps the cell to survive adverse environmental conditions (Audia et.al., 2001). . The parameters for survival include glucose consumption (Adenosin Triphosphate, ATP), proton concentration, temperature, anaerobic conditions. Death occurs when energy sources (pH, salt etc.) within the water droplet are depleted to a level lower compared to adaptation mechanism of the stress regulon factors. Table 2.1summarises aW ranges for various microorganisms that is optimum for their growth.

The Food Standards Agency (FSA) UK in February 2009 launched a public health campaign to raise the awareness of health risks of eating too much saturated fat. The UK population currently consumes 20% more saturated fat than the Government recommendations. The Agency has also revised salt reduction for 80 categories of foods to ensure food retailers and manufacturers maintain the momentum in reducing salt levels. The revised targets also reflect the Agency's long-term commitment to reducing people's average salt intake to 6g a day. The

previous targets for 2010 were set in 2006. Many manufacturers and retailers have made considerable reductions in salt levels. However, salt levels still vary considerably between different products. The salt reduction targets have been set for foods that make the greatest contribution of salt to our diet, such as bread, meat products and cereals, as well as convenience foods such as pizza, ready meals and savoury snacks. About 75% of the salt we eat is already in the food we buy. Hence theFSA has issued a new target for the manufacturers to produce product with reduced salt level and less saturated fat (Table 1.7) (www.fas.gov.uk,Mar 2014). Salt and fat gives the product an extra microbiological growth hurdle from microbial spoilage or poisoning. Therefore this will be an added danger to the product. The 2017 targets recognise the progress that has already been made by the food industry and aims to encourage further reduction. Salt levels in many foods covered by previous targets have reduced significantly, some by 40-50% or more, and more than 11 million kilograms of salt have been removed from foods (www.fsa.gov.uk, Mar 2014). However, average UK salt consumption remains high at approximately 8.1g-8.8g/day so there is still considerable reduction required to meet the 6g/day population intake goal for adults and less for children. The 2017 targets are suitably challenging and wide-ranging but recognise the different starting points and technical challenges. The 2017 categories remain substantially the same as the previous 2012 targets with an additional category added meat extracts; one sub-category on salted butter was removed where industry had already met the target; and several minor changes have been made to better reflect current market position.

In spreads, the salt concentration can be up to 8% in the aqueous phase. The sodium chloride level may vary from 0 to 2% w/w although sometimes it can be as high as 2.5% in the finished product (which corresponds to up to 11% in the aqueous phase). Sufficient addition of salt to the water phase should stop bacterial growth. Use of salt at 1% prevents the growth of the majority of microorganisms and at 2% the prevention is total. On the other hand products with salt concentrations of 0.1 –0.2% support the growth of microbes (McSweeney 2009).

Table 1.7 FSA 2017 salt reduction target (FSA, 2014)

1.1.4.5 Lactic Acid Bacteria

As per Codex regulations (Codex Stan A-1-1971, Rev.1-1999, Amended 2003) Lactococcus lactis subspecies lactis and cremoris can be added to the spread formulation as a processing aid for cream flavour and maturation rather than an additive. But the droplet size will be a limiting factor here, hence the finer the droplet size the greater concentration of the starter culture will be required.

1.1.4.6 Moisture Droplet Size

The spread preservation system depends on the presence of a fat-continuous matrix. Any water present is in the form of very finely dispersed droplets. Due to this dispersion, contaminating microorganisms are restricted in growth either by space limitations or by exhaustion of the nutrients in the droplets. Shells of fat crystals covering water droplets prevent these from merging together (Juriaanse and Heertje, 1988). Droplet sizes can be estimated by microscopic observation,

although this technique is not sufficiently accurate to describe the population of droplet sizes in quantitative terms.

In a ‘water-in-oil’ product, the water is present as well-dispersed fine droplets throughout the fat phase. The inability of microorganisms to move between droplets is a major intrinsic preservation factor (ICMSF, 2005). Fat can act as a barrier to microbial growth, resulting in much more stable fat continuous systems than water continuous systems. This generally limits microbial growth, but does not necessarily exclude growth under extreme conditions (ICMSF, 2005). The ability of a microorganism to grow in an emulsion depends partly on the volume of the water droplet it finds itself in (Charteris 1995). The mean diameter of the droplet size in the aqueous phase of a spread is usually between 4 and 5 μm, but can range between 1 and 100 μm (Charteris 1995). Microorganisms cannot survive in emulsions with a droplet size of less than approximately 5μm, but can sustain if it is within the range of 5-10 μm (Boysen 1927). The number of the droplet size is dependent on the percentage of the aqueous phase and the interstitial space between the droplets. The dimension of bacteria ranges from 0.4 – 1.5 X 0.5 -5 μm for bacilli or approximately 0.5 – 2 μm for cocci. Alive bacterial cells are not detected in below ≤ 10 μm (Charteris 1995). The distribution for yeast and mould (Y&M) is considered to be similar (Charteris 1995) they can survive in in droplet size ≥ 30 μm. But coalescence of droplets can release water at the product surface allowing mould growth.

Water droplets are larger in products containing less fat, thus providing cells with increased space and more water-soluble nutrients from the ingredients. Temperature, redox-potential and pH influence the energy-dependent growth of microorganisms in water in oil emulsions.

1.2 THE PRODUCT CHARACTERISITICS

The UK dairy industry with the Red Tractor logo is proud of their links to the countryside, heritage and the part they play in everyday life. Their vision is usually to earn the right to consumer's loyalty by providing healthy, enjoyable and convenient products and aim to meet consumers' needs.

The Red Tractor Code of Practice for t quality assurance outlines Product Control standards and controls to ensure all products produced comply with food safety, legal, quality requirements and meet consumer expectations along with the control of Non-Conforming Products, Quality Monitoring Plan, labelling. These are for any Food Business Operators (FBO) management system.

To ensure product quality as per the Quality Codes of Practice, specific attention is given to the prevention and control of pathogens during the production process itself and then the shelf life of the product. Relevant pathogens are evaluated during the product design, e.g. infectious pathogens, such as Salmonella, Escherichia coli, and Listeria monocytogenes. Various indicator organisms are used as process and hygiene indicators such as Enterobacteriaceae and

Staphylococcus aureus that will highlight any process or environment cross contamination early in the process.

Factors needing particular attention to achieve a safe product are listed below:

1. Initial microbiological contamination of the raw materials and ingredients
2. Microbiological stability of the product formulation
3. Pasteurisation step(s) during manufacture
4. Post Process Contamination (Cleaning, Environment as water and air, packaging)
5. Shelf life conditions (time, temperature)

Raw materials that may be susceptible to contamination with pathogenic microorganisms are added to the product formulation at the pre-pasteurisation step. This is ensured by effective supplier approval and monitoring, that will ensure any potential risks posed to the safety, legality and quality of the final product by suppliers are understood and managed. The supplier assurance covers all raw materials, ingredients, packaging, service providers and contingency providers for all FBOs including the co-packers.

1.2.1 Microbiological Stability

Microbiological stability may be defined as the degree to which a formulation allows growth of relevant microorganisms at ambient temperature (both pathogenic and spoilage micro-organisms) if it has been exposed to contamination. Microbiological stability of a product depends on chemical composition (e.g. pH, preservative, and salt) as well as nutritional (e.g. lactose,

whey) and physical factors (water content, emulsion characteristics: droplet size (average size, total range of sizes). Since most spreads are water-in-oil emulsions it requires a very important preservation principle of the aqueous phase being dispersed as droplets in a fat-continuous phase. As these droplets are limited in size, any microorganism, if it were to contaminate the product, would be restricted in its growth either due to space limitation or due to depletion of the nutrients available in the droplet in which it is present (McKenna 2003). A hazard category may be given to a group of products based on their microbiological stability. Hazard categorisation facilitates the provision of hygiene rules and shelf life conditions (Klapwijk 1992).

Consumers recognise mould spoilage as a defect in fat products. Behaviour of moulds is quite unlike that of bacteria and yeasts (Pitt 2009) and a separate estimation of mould vulnerability may be required as part of product development. Fat products have a limited shelf life once opened, due to the potential for mould spoilage. Preservatives such as sorbates reduce the risk of mould problems if used at the right concentration and pH (≤ 5.5) (Marin et al, 2003).

1.2.2 Pasteurisation steps during manufacturing

The aim of product pasteurisation is to eliminate all infectious pathogens and to reduce the number of spoilage microorganisms to an acceptable level. Bacterial spores of toxigenic microorganisms (e.g. Bacillus cereus) cannot be eliminated by pasteurisation because of their heat resistance. For these microorganisms it is

important that growth in the final product is either prevented or controlled to an acceptable level by the product composition and/or emulsion characteristics and/or the shelf life conditions. Although commonly produced fat product formulations do not allow growth of these organisms at the relevant shelf life conditions (Rajah 2002), in view of new consumer demands (including milder tasting products, absence of preservatives), these micro-organisms may need more consideration in new product development.

The company pasteurisation conditions are typically 15-20 sec at 72ºC for liquid products (company manufacturing standard); this pasteurisation is not considered adequate for spreads because of percentage of fat content within the product, even though main ingredients as cream and buttermilk are already pasteurised.

Pasteurisation conditions for emulsions for the company is set at a minimum of 2 minutes at 80ºC or equivalent (company manufacturing standard). At low microbial load of the raw materials, in practice this treatment will also be sufficient to control spoilage. As has been mentioned for the pasteurisation of aqueous solutions, some special products (e.g. low a_w due to high salt-in-water content >8%, or products where fermented raw materials are used) may require a more severe heat treatment (Rajah 2002; Andrew 2003; Ranieri 2009).

1.2.3 Chemical Contaminants

Chemical contaminants can originate from several sources such as natural sources (e.g. aflatoxin), cleaning agents, transport etc. Appropriate measures should be

taken to eliminate or substantially reduce this risk. Ways of reducing these risks can be selection of material suppliers, transport, storage and adherence to cleaning instructions.

1.3 SPREAD SPOILAGE

1.3.1 Lipolysis in Spreads

As spreads become rancid (undergo breakdown of the fat), components are produced which may give rise to changes in both the physical and organoleptic properties of the product. In general the liberated free fatty acids and peroxides thereof are of most interest and are measured relatively easily within the laboratory. However the fatty acids can be broken down still further into smaller volatile molecules such as aldehydes and (methyl) ketones (Stead 1986; Allen and Hamilton 1994; Brocklehurst 1995). Many of the fat breakdown products have characteristic odours and can give rise to unpleasant taints within the product making it undesirable to the consumer. Of relevance to this study are the methyl ketones since these give rise to ‘cheese flavour’ cheese odours within the product that can be detected at low levels organoleptically.

One of the routes of product breakdown is contamination by microbiological organisms (Aprigny and Jaeger 1999). Certain groups of microorganisms can be characterized by the way they metabolize the product fat and break it down (Allen and Hamilton 1994; McKenna 2003). The measurement of specific breakdown products such as methyl ketones can be a useful tool in predicting the nature of

microbiological contamination. The relatively rapid analysis times compared to the microbiological analysis allows preliminary investigation to proceed pending microbiological confirmation.

The main organisms that may cause lipolytic microbial spoilage or product quality issues are listed in Table 1.8. Pseudomonas spp causes black, green or metallic discolouration of the product followed by fruity or metallic taints (Allen and Hamilton 1994). Bacillus spp may cause black discolouration and result in ropiness as a sign of spoilage as the spores start germinating at 4ºC (Allen and Hamilton 1994; McKenna 2003). The presence of lipase in Micrococcus spp can produce heat stable lipases and moulds show visible growth in the product and they contain reactive lipases and free fatty acid (FFA) oxidative enzymes. Yeasts are usually salt and acid tolerant and hence are one of the main organisms that causes quality issues (Allen and Hamilton 1994; McKenna 2003).

Table 1.8 List of possible lipolytic organisms in dairy products (Allen and Hamilton 1994; McKenna 2003)

Type of Microorganism	Organism	Heat Resistance
Bacteria	*Bacillus* spp (spore former)	Can survive HTST
	Micrococcus spp	
	Pseudomonas spp	Destroyed by pasteurisation
	Bacillus spp (vegetative form)	
Mould	*Penicillum* spp	
	Aspergillus spp	
Yeasts	*Candida* spp	
	Saccharomyces spp	

1.3.2 Methyl ketones

Milk triglycerides contain a much higher proportion of short chain acyl groups and the C4 – C8 fatty acids have their own characteristic taste. 'Cheese flavour' is composed of four main methyl ketones, formed from small to medium chain saturated fatty acids (Table 1.9) (Patton 1950). Dairy products inherently have a low level of methyl ketone where no undesirable side effect is noted (Table 1.10).

The simplified mechanism of lipolysis that may result in the distinctive ’cheese flavour’ smell is illustrated in Figure 1.10. Lipolysis is the hydrolysis of the triglycerides to product FFA, which possess an undesirable flavour. Most of the lipolytic rancidity in the dairy product occurs by the enzyme lipase (Allen and Hamilton 1994). FFA is measured by titration, where the fat is extracted and dissolved in a neutralizing solvent (Patton 1950).

In good quality microbiologically clean milk, the lipolysis is usually due to the intrinsic milk lipoprotein lipase. High FFA levels can arise either from high levels in the milk used, pre-manufacture lipolysis or post pasteurisation lipolysis (Allen and Hamilton 1994). The latter mainly is caused by microbial contamination during or after processing or by contamination of heat resistant enzymes from psychrotrophic bacteria that are able to grow in storage silos below 5ºC (Chavez 1994). Therefore the problem may often be intermittent and difficult to trace.

Table 1.9 Four methyl ketones responsible for 'cheese flavour' (Patton 1950)

Methyl Ketone	Length of Carbon Chain	FFA Derived from	
		Systemic Name	Traditional Name
2-pentanone	5	Hexanoic Acid	Caproic Acid
2-heptanone	7	Octanoic Acid	Caprylic Acid
2-nonanone	9	Decanoic Acid	Capric Acid
2-undecanone	11	Dodecanoic Acid	Lauric Acid

Table 1.10 Free Fatty Acid concentration in dairy products (Allen and Hamilton 1994)

Product	FFA Values (meq/100g fat)	
	Normal Values	Likely to cause problem
Milk	0.3 – 1.0	1.5 – 2.0
Cream	0.5 – 1.2	1.7 – 2.1
Butter	0.5 – 1.0	1.0 – 2.1
Cheddar Cheese	1.2	-
Cheese flavour	40	-

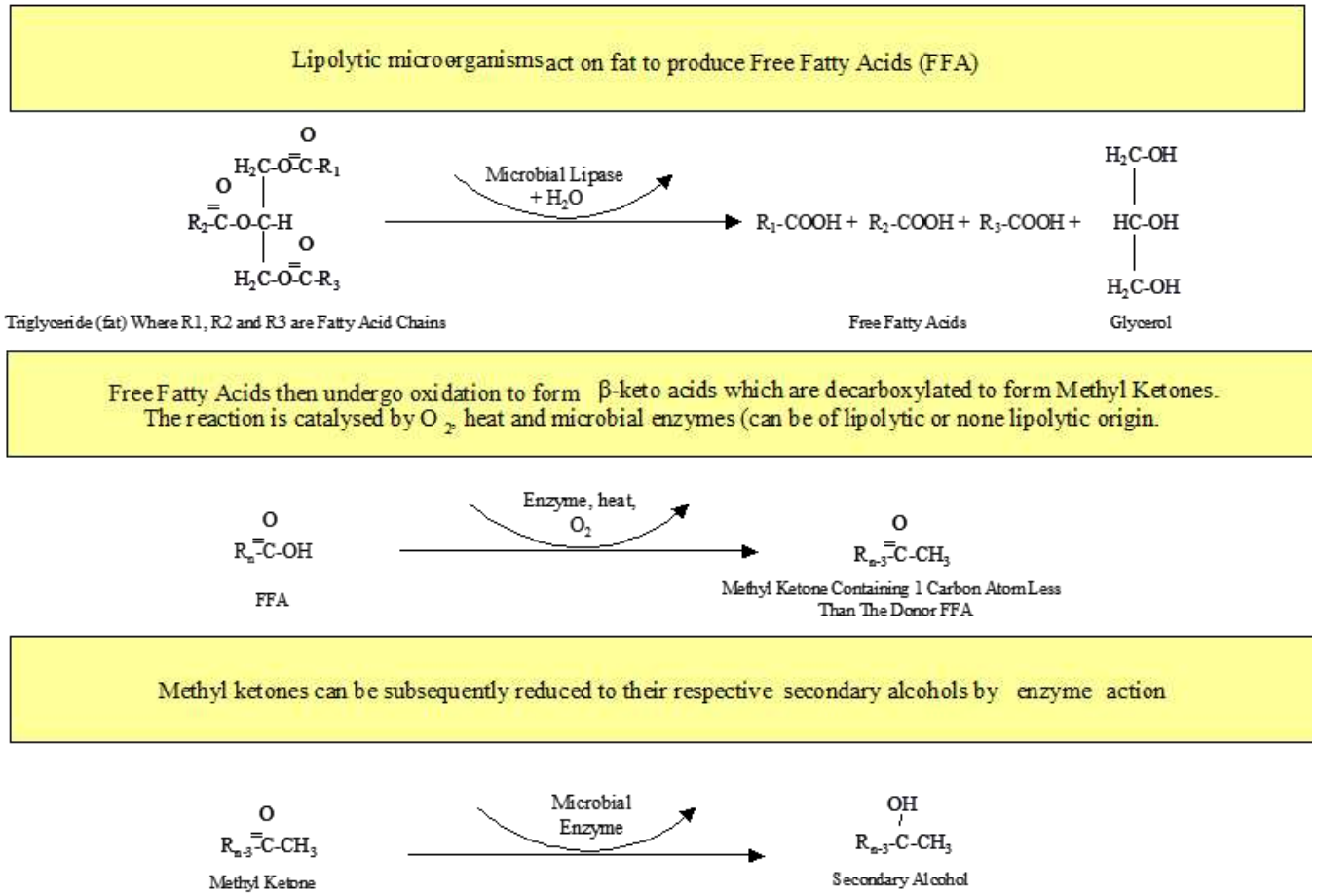


Figure 1.10 Simplified mechanism of lipolysis and production of methyl ketones (Allen and Hamilton 1994).

CHAPTER 2 SPREAD PRODUCT HURDLE AND EPIDEMIOLOGY

The concept of ‘Product Hurdle Rate’ ensures that products have food safety and spoilage considerations built in or engineered out prior to launch and to determine whether any changes to the site HACCP study are required. This also highlights the requirement for shelf life study and microbiology challenge testing. The hurdle profile gives overall guidance on the risks associated with the product and its development, for example the product itself, the processing site and the processes involved. A multidisciplinary team should be involved in developing the risk profile highlighting the following:

- A clear guidance on the product development timeline (if the timeline is practical)
- Any benchmark product in the market.
- Manufacturer and Competition
- Compliance with all the relevant requirements.

Figure 2.1 shows a process as to how the product hurdle rate can be generated based on the product intrinsic factors such as droplet size, pH, salt, moisture, water activity and the presence of antimicrobials. The above factors reflect the product spoilage or degradation rate, which then forms several unpleasant biochemical substrates in the product.

At the end of every production run in a butter or spread manufacturing site all the equipment with direct or indirect product contact such as the silo, churn and filler have adherence of product to the stainless steel surface due to the high content of fat. Hence a cleaning programme will look into both the removal of gross soil removal and destruction of microorganisms on the surface (Stall 1986). The factory can be cleaned in two ways, manual for open equipment soil removal and an automated via Cleaning In Place (CIP) system for the enclosed vessels and pipe work. No product recycle was involved in the process.

CIP consists of various stages of cleaning such as rinsing, caustic circulation (1% caustic soda at 80ºC for 45 min), rinsing followed by acid circulation (0.5 – 1% acid at 70ºC for 45 min) followed by a final rinse (Stall 1986). At the rinsing step the system is flushed with cold water to flush out any gross debris followed by a detergent circulation. Caustic and acid cleaning sterilises the equipment from organic soils along with de-scaling to remove inorganic soils that prevents any biofilm formation. The cleaning is further validated and verified with ATP and microbiology swabs (CCFRA guideline NO 55 (2008).

A certain number of organisms do survive irrespective of the robustness of known production processes. The objective of any research on quality management of these products is minimization of the number of these organisms. High numbers denote either poor raw material selection or poor production process hygiene or both.

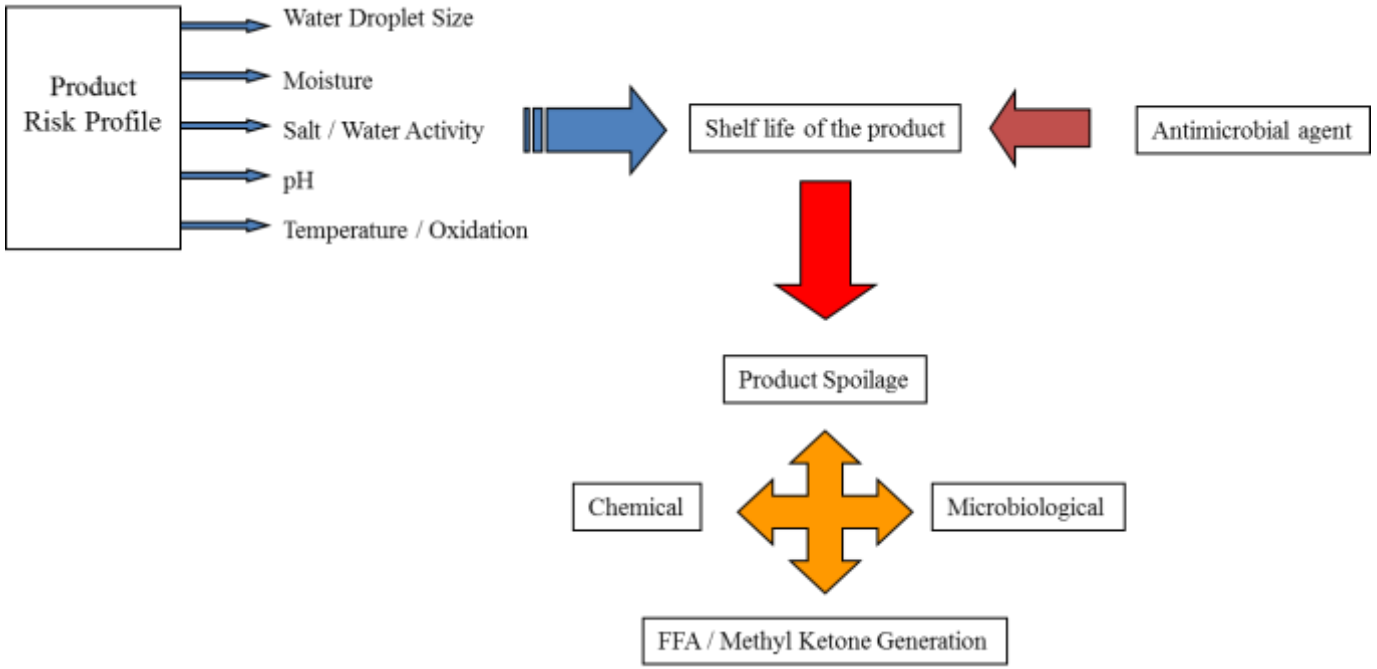


Figure 2.1 Product hurdle profile matrix

Many spreads may allow the growth of yeast, mould and spoilage bacteria (e.g. Enterobacteriaceae, Pseudomonads, and aerobic spore formers) and even pathogenic bacteria when present or introduced during production / open shelf life. Most frequently spoilage of butter and margarine are caused by moulds affecting the product quality (Hocking 2009). Preservatives such as benzoate, sorbate, salt, pH and water droplet size greatly reduces mould problems during distribution and consumer use. Table 2.1 summarises the minimum growth requirements for various microorganisms as per Campden BRI guidelines No 28 (2007).

Reduced fat spreads are more vulnerable to microbiological problems, this may be counteracted by pasteurisation and use of preservatives or increased attention to process line hygiene and equipment design. The microbiological stability of the complete formulation will determine the hygienic requirements of the production process (Stall 1986). Growth of microorganisms before pasteurisation should be prevented because of metabolic products (enzymes, off-flavours, toxins). Equipment should be designed to prevent contamination during processing especially after the last decontamination step.

Proper hygiene during packing is also important especially with respect to contamination by moulds. Post process temperature abuse must also be considered as this may cause condensation of moisture on the surface of the spread and destabilization of the physical product structure. Both factors can compromise the preservative effects of the compartmentalized structure allowing

potential cross contaminants to grow. Challenge testing may help to assess the robustness of formulations (Campden Challenge Test Protocol Guideline No 63, 2010).

For the food safety and commercial quality of spreads, two types of microorganisms are of concern, pathogenic (L. monocytogenes, E.coli, Bacillus spp, Salmonella spp. etc.) and spoilage (Y&M) (Charteris 1996). The general food industry usually screens for indicators in the product and environment as guidance to the indicator of hygiene, cleanliness and pathogens as it impractical to test food (off-line or on-line) for all the microorganisms (EU Micro Criteria 2073:2005). The general indicator counts used across the food industry as per the EU Micro Criteria are Total Viable Count and Enterobacteriaceae family and in the presence of their increased numbers further screening for E.coli and Listeria spp is carried out. The detection of the indicator organisms does not suggest the presence of pathogenic or spoilage organisms but shows that an appropriate environment and food source are present for them to grow (Charteris 1996). As per the Codex guideline and EU Micro Criteria, the main microbial groups of concern microorganism in edible spreads are Enterobacteriaceae or the Coliform family, Y&M, Bacillus spp, S. aureus, Listeria spp, Salmonella spp.

Table 2.1 Growth Limits of Pathogens (Industrial Microbiological Risk Assessment Guideline No. 28 Campden BRI)

Microorganisms	Minimum Requirements			
	Temp (°C)	pH	a_w	O_2
Mesophilic *B. cereus*	15	4.3	0.95	Facultative
Psychotrophic *B. cereus*	4	4.3	0.95	Facultative
C. jejuni	32	4.9	0.99	Microaerophilic
Mesophilic *Cl. botulinum*	10	4.6	0.93	Anaerobic
Psychotrophic *Cl. botulinum*	3	5.0	0.97	Anaerobic
Cl. perfringens	12	5.0	0.95	Anaerobic
E. coli	7	4.4	0.95	Facultative
E. coli 0157	6.5	4.5	0.95	Facultative
L. monocytogenes	0	4.3	0.92	Facultative
Salmonella spp	7	4.0	0.94	Facultative
Staph. aureus	6	4.0	0.83	Facultative
V. parahaemolyticus	5	4.8	0.94	Facultative
Y. enterocolitica	-1	4.2	0.96	Facultative

2.1 LISTERIA MONOCYTOGENES

Listeria monocytogenes is a Gram-positive, short, non-spore forming rod, typically of 2 µm in length and 0.5 µm in diameter, is motile at 25°C (tumbling motility 20-22°C) but is non-motile at 35°C. It is a facultative anaerobe and is also psychrotrophic. Occasionally it produces a coccoid form or individual cells which are typically 10 µm in length. It is catalase positive and oxidase negative, and fermentative. Six species of Listeria are recognised. Almost all cases of human listeriosis are reported to be due to Listeria monocytogenes. Almost all L. monocytogenes are β haemolytic on blood agar (Jay 1996).

L. monocytogenes is widespread in the environment and sporadically found in ready to eat (RTE) foods in low numbers. EU regulations suggest that in RTE food that are able to support the growth of the organism, it should be absent in 25g food (EU Micro Criteria 2073:2005). It has been isolated from a wide variety of habitats including soil, sewage, water and the faeces of healthy humans and animals. Faecal material of healthy grazing animals can be contaminated with L. monocytogenes and the frequency of isolation from faeces can be affected by diet, particularly when feeding on poor quality silage (Kelly 1996; Fenlon 1999; Kelly et al. 2000).

2.1.1 Human Listeriosis

L. monocytogenes causes various conditions in both man and animals, including septicemia, encephalitis and abortion in farm animals and humans and necrotic hepatitis in poultry. The most common forms of human listeriosis are as meningitis or septicemia either in immunocompromised adults or juveniles or unborn infants. Listeriosis can be transmitted by direct contact with the environment and by contact with infected animals or animal material, although these routes are rare. Healthy humans carrying L. monocytogenes have not been identified as a source of the organism for cases of listeriosis (Jay 1996).

The incubation period varies widely between individuals from 1 to 90 days, with an average for intra-uterine infection of around 30 days (McLauchlin 1996a). Listeriosis in pregnancy manifests itself as severe systemic infection in the unborn or newly-delivered infant as well as a mild influenza-like bacteraemic illness in the pregnant woman. Infection can occur at any stage of pregnancy. Listeriosis in children older than 1 month is very rare, except in those with underlying disease. In adults and juveniles the main syndromes are septicaemia and/or central nervous system infection. Most cases occur in immunosuppressed patients receiving steroid or cytotoxic therapy or with malignant neoplasms. However, about one-third of patients with meningitis and around 10% with primary bacteraemia are immunocompetent (Smerdon et al. 2001). The mortality rate in systemic listeriosis is relatively high for a foodborne pathogen and can vary between 20-40% (McLauchlin 1993; Rocourt, 1994; Farber et al. 1996) and survivors,

particularly those where the organism has invaded the central nervous system, can develop serious long-term sickness.

Foods associated with acute gastroenteritis with fever outbreaks have been diverse, but heavily contaminated by the bacterium (Aureli et al. 2000). Symptoms typically develop in 1–2 days. Large numbers of L. monocytogenes are present in the stool, and a few cases develop serious systemic infection. The ability to cause gastroenteritis may be specific to certain strains. Rarer manifestations of listeriosis include arthritis, hepatitis, endophthalmitis, endocarditis, cutaneous lesions, pneumonia and peritonitis in patients on continuous ambulatory peritoneal dialysis. A threefold increase has been observed since the year 2000 especially in the elderly category with increasing high mortality rate (ACMSF 2005).

Listeria spp outbreak has been noted on various dairy products made from raw milk as milk, butter, spreads and cheese, but not from products made of pasteurised milk or cream.

2.1.2 Infective Dose

The wide distribution of L. monocytogenes and its ability to grow at low temperatures indicates a high frequency of exposure for humans. Despite this high frequency, human listeriosis is relatively rare. This may be due to the fact that illness develops as a result of the interaction between host susceptibility, pathogenicity of the microorganism, and type of contaminated food consumed.

The interaction between these factors affects the infective dose for L. monocytogenes. The infective dose for systemic infection is not known, however this is likely to vary widely between different individuals. Studies on monkeys (Farber et al. 1991) and mice (Golnazarian et al. 1989), suggest that reduced levels of exposure will reduce clinical disease. Such data cannot however, be extrapolated to humans and the debate on infective dose remains open. Food remnants available from cases of listeriosis have generally been contaminated with high levels (e.g. >1000 cfu/g food) of L. monocytogenes (McLauchlin 1996a). However, the limited number of cases for which information on the levels of L. monocytogenes in the incriminated food, the degree of variability in the immune status of the patients, and the variable virulence of different strains, do not exclude the possibility that infective doses might be lower than 100 cfu/g (McLauchlin 1996a; Rocourt 1996).

2.1.3 Effect of Salt Concentration

Growth at low temperatures is affected by salt concentration (Schaack and Marth 1988a,b,c). The maximum concentration of salt (NaCl) allowing growth is about 12% w/w. Interactions with sodium chloride, temperature and pH show growth was recorded in the presence of 10% NaCl at 25^{o}C at pH of 5.0 after 41 hours at pH of 6.0 and 7.0 after 31 hours and pH 8.0 after 35 hours (McClure et al. 1989). Voysey (2009) in his study reported that L. monocytogenes cells can elongate up to 55 μm in length and become filamentous in 8-9% NaCl when grown at 30 or 37^{o}C, whereas cell multiplication is inhibited under these conditions (Isom et al. 1995). At 30 or 37^{o}C, filament formation takes place above 1000 mM NaCl and

peaks at 1200-1500 mM. This effect is also induced in the presence of citric acid at pH 5-6 and media supplemented with 175 mM H_2O_2 (Isom et al. 1995).

2.1.4 Growth in Fat

L. monocytogenes favours the aqueous rather than the lipid phase in foods such as butters. Fats can allow survival of L. monocytogenes cells when they are heat-treated at some pasteurisation temperatures (Fain et al., 1991).

2.1.5 Effect of Preservatives

Growth of L. monocytogenes is best controlled by a combination of effects such as temperature and preservatives acting together (Connor et al.1986; Buchanan et al. 1989; McClure et al. 1989, 1991; Buchanan and Phillips 1990; Cole et al. 1990; Wijtzes et al. 1993). Nisin and other bacteriocins produced by lactic acid bacteria are useful in the control of Listeria (Dean and Zottola 1996). Validation by food manufacturers of the effects of preservative systems in their product is extremely important, due to any potential partitioning effects of particular preservatives/antimicrobials in the product phases. It is well established that some preservatives, such as weak acids are able to partition into the lipid phase of foods, thereby reducing their aqueous phase concentration and consequently to act as preservatives.

2.1.5.1 Effect of Sorbate

In combination with low pH, sorbate will inactivate L. monocytogenes, e.g. a 4-5 log reduction at pH 3.3 after 4-8h at 30^{o}C (Corte et al. 2004). At higher pH values, sorbate will inhibit growth of L. monocytogenes by at least 2 log cycles (as compared to the unpreserved control) at the end of storage of 8 weeks (Choi and Chin 2003). Also addition of 1% potassium sorbate to cheese brines decreases survival of L.monocytogenes (Larsen et al. 1999), inhibits growth in beaker sausage (Hu and Shelef 1996) and other products (El Shanawy and Marth 1988, 1991; Buazzi and Marth 1991; Wederquist et al. 1994; Buncic et al. 1995). In broth, minimum inhibitory concentrations of 400-600 mg/l and >5000 mg/l were reported at pH 5 and 6 respectively at 35^{o}C, by Moir and Eyles (1992). At low temperatures (5^{o}C), the MIC at pH 6.0 reduces to 1500 mg/l. Sorbate works best at pH values below 6.0 and is not practically effective at pH values of 6.5 and above.

2.1.5.2 Effect of Lactate

The concentrations of lactate required to inhibit growth of L. monocytogenes are usually around 2-4% in foods. As with other preservatives, inhibitory effects are enhanced at lower temperatures of storage and also in combination with other factors such as nitrite and salt (NaCl). For example, in the presence of 2% NaCl, 2-3% lactate inhibited growth of L. monocytogenes whereas 4% was required if lactate was used alone (Chen and Shelef 1992). Using combinations of NaCl (3% in the aqueous phase) and lactate (2%) and storage at chill temperatures can result

in very long shelf-lives (e.g.>50 days) for some products such as smoked comminuted vacuum-packed salmon (Pelroy et al. 1994).

2.1.5.3 Effect of Benzoate

In a study by El-Shanawy and Marth (1988), it was reported that sodium benzoate is more inhibitory to L. monocytogenes than potassium sorbate or sodium propionate at a concentration of 2000ppm sodium benzoate after 60 days at 4^{o}C, at pH 5.6, and 1500 ppm after 24 days at 4^{o}C at pH 5.

2.1.5.4 Effect of Propionate

In the same benzoate study a by El-Shanawy and Marth (1989) showed that >2000 ppm sodium propionate inhibits growth of Listeria spp at pH 5. El-Shanawy and Marth (1992) also showed that organic acids such as acetic, tartaric, lactic or citric acids significantly increase the inhibitory effects of propionate on the growth of L. monocytogenes.

2.1.6 Incidences

The majority of cases of human listeriosis in RTE refrigerated foods in UK that are capable of supporting the growth of the bacterium are sporadic (ACMSF 2001), with a reported increase since 2000. An increased number of cases have been reported exclusively in patients aged over 60 years presenting with listerial bacteraemia within the UK and European countries. An incidence of listeriosis of

7.1 cases per million of the population in the United States was estimated in the late 1980s compared to in estimated between 0.1 and 11.3 cases per million of the population in Europe (Voysey 2009). The recent rise in listeriosis in England and Wales has resulted in a maximum rate of 4.5 cases per million in 2003 down to 3.5 cases per million in 2005 and 2006 (FSA 2005).

In the UK, two outbreaks of listeriosis in 2004 occurred due to contaminated butter production that spread from the factory to retail, and catering premises in the United Kingdom during May and June 2004 (Lewis et.al. 2006). As per EU Micro criteria 2073:2005 for RTE foods that are able to support the growth of L. monocytogenes, it should be absent in 25g food. In the butter samples tested, 99.4% of samples were found to be of satisfactory quality, 0.5% were of acceptable quality, and 0.1% were of unsatisfactory quality as a result of high levels (>100 CFU/g) of Listeria spp. The butter samples with Listeria spp present at more than 100 cfu/g were negative for L. monocytogenes. L. monocytogenes was detected in 0.4% (n=13) of samples, all at levels of less than 10 cfu/g in enumeration that equates it to being absent as while testing one dilution is performed for butter. In another study by Bille et al. (2006) a large national retailer sold butter which was produced in a cheese factory and was only sold within the local area. Five out of 10 samples from two different lots were found to contain L. monocytogenes and therefore could not be sold, according to EU Micro criteria. The levels detected in butter were ≤ 100 cfu/g. Table 10.1 Appendix 10.2 summarises the outbreaks of pathogenic microorganisms in UK and Europe in milk and milk products since 2010, showing no major outbreak was observed in

the butter and margarine category with L. monocytogenes (RASFF alert https://webgate.ec.europa.eu/rasff-window/portal/).

2.1.7 Importance in Food

The consumption of contaminated foods is the principal route of transmission of L.monocytogenes. The organism can survive for many weeks in frozen foods. Microbiological and epidemiological evidence supports an association with many food types (e.g. dairy, meat, vegetable, fish and shellfish) in both sporadic and epidemic listeriosis. Foods associated with transmission often show common features (McLauchlin 1996a):

- The ability to support the multiplication of L. monocytogenes (relatively high water activity and near-neutral pH);
- Relatively heavy contamination (>1000 organisms per g) with the implicated strain;
- Processed, with an extended (refrigerated) shelf life;
- Consumed without further cooking.

2.2 E.COLI

E. coli is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm blooded organisms (endotherms). Most E. coli strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls. E. coli and related bacteria constitute

about 0.1% of gut flora and faecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for faecal contamination (Jay 1996).

Most E. coli infections come from:

- Eating undercooked ground beef (the inside is pink)
- Drinking contaminated (impure) water
- Drinking unpasteurized (raw) milk
- Working with cattle

Healthy beef and dairy cattle may carry the E. coli bacterium in their intestines. It can also be passed from person to person through farm or slaughter house hygiene (FSA 2009).

2.2.1 Pathogenicity and Virulence

Enteropathogenic Escherichia coli (EPEC) remain an important cause of diarrhoeal disease worldwide. The pathogenesis of EPEC depends on the formation of an ultra-structural lesion in which the bacteria make intimate contact with the host apical enterocyte membrane the ‘attaching and effacing mechanism’ (Holliday et.al, 2003). The formation of this lesion is a consequence of the ability of EPEC to adhere in a localized manner to the host cell, aided by bundle-forming pili. Tyrosine phosphorylation and signal transduction events occur within the host cell at the lesion site, leading to a disruption of the host cell mechanisms and,

consequently, to diarrhoea. These results from the action of highly regulated EPEC secreted proteins that are released via a type III secretion system, many genes of which are located within a pathogenicity island known as the locus of enterocyte effacement eae (Jay 1996).

E. coli 0157:H7 is a pathogenic strain of E. coli that was first identified as a cause of disease in the United States in 1982, during an investigation into an outbreak of gastrointestinal illness. The organism is also known as enterohemorrhagic E. coli (EHEC) because it causes abdominal pain with diarrhoea that may contain blood. It lives in the intestines of healthy cattle and can contaminate meat during slaughter. The verotoxins that cause the disease are also called shiga toxins because they were acquired from Shigella strains. The illness predominantly occurs in the U.S., Canada, Japan and other industrialized nations of Europe. The average incidence in most countries ranges from 1-30 per 100,000 and the number of confirmed cases is increasing (FSA 2012).

EHEC travels through the digestive system and attaches to the mucosal epithelial cells of the large intestine and forms a pedestal, which leads to destruction of the microvili. The bacteria will multiply and produce toxins, which, in addition to diarrhoea cause vomiting in 50% of cases and fever in 30%. The average incubation period is 3-4 days. The inoculum size is small (as little as 50 cells can cause illness) because E. coli 0157:H7 are acid tolerant and can easily pass through the stomach. The genome of E. coli 0157:H7 was recently sequenced and contains 1,387 new genes absent in non-pathogenic strains. Analysis of the

genome suggests that there are a variety of potential virulence genes that have yet to be explored, including fimbrial and other adhesions, secretion systems, and toxins. Many of the virulence genes are similar to those associated with pathogenesis in Salmonella and Shigella (Jay 1996).

Outbreaks of E. coli 0157:H7 are almost invariably traced to foods of bovine origin. Other foods that have been implicated in the spread of E. coli 0157:H7 include apple juice and cider, milk, cheese, yogurt, soy beans, lettuce, tomatoes, other fruits and vegetables, and basically anything grown on or near herds of cattle. Contaminated water sources such as lakes, ponds and reservoirs have also been linked to outbreaks (FSA 2012).

Enteroinvasive Escherichia coli (EIEC) cause bacillary dysentery in humans by invading and multiplying within epithelial cells of the colonic mucosa. The pathogenesis of the potentially life-threatening diarrhoea caused enterotoxigenic Escherichia coli (ETEC) and produces cholera like toxin. Their outbreaks are more water related compared to bovine outbreaks as EPEC and EHEC (Jay 1996). On the other hand, Enteroaggregative Escherichia coli (EAggEC) are diarrhoeal pathogens defined by aggregative adherence to HEp-2 cells. A study by DEFRA in 2004 showed a lot of the healthy animals at slaughter in UK can carry EAggEC, but when they were exposed to specific EAggEC probes the results were negative. Only EHEC is routinely carried by animals and the rest are water borne and hence only infrequently associated with foods.

2.2.2 Infective Dose

The infective dose of E. coli is uncertain. Some strains of E.coli can be harmful as low as 10 cfu /ml for EHEC, whereas for the rest of the other strains can be as high as 1000 cfu / ml. The presence of Enterobacteriaceae in product or environment above the level of 100 cfu / g / or swab is a good indicator of poor hygiene (Public Health England 2009).

Symptoms by EHEC include abdominal cramps, diarrhoea and occasionally fever and vomiting. . The incubation period can range from three to eight days followed by a 10 day recovery period but may vary depending on the population. In children and elderly it may lead to a life-threatening disease, such as haemolytic uraemic syndrome (HUS), characterized by kidney failure. It can cause neurological complications (such as seizure, stroke and coma) in 25% of HUS patients and chronic renal sequelae, usually mild, in around 50% of survivors (FSA 2014). DNA from a Shiga toxin producing bacterium known as Shigella dysenteriae type 1 is known to transfer to E.coli via bacteriophage and regulated production of potent shiga toxin (Jay 1996).

2.2.3 Effect of Salt Concentration

E.coli can grow without salt present but can also tolerate small amounts of salt in its growth medium. E.coli may have optimal growth in the absence of salt, but in the presence of salt it will grow, just at an attenuated rate (Holliday 2003). Growth in environments with a wide range (at least 100-fold) of osmolarities poses significant physiological challenges for cells. To meet these challenges, E. coli

adjusts a wide range of cytoplasmic solution variables, including the cytoplasmic amounts both of water and of charged and uncharged solutes (Fisher 1998, Voysey 2009).

2.2.4 Growth in Fat

Commercially available emulsions of 10% and 20% soybean oil and 10% sunflower have shown the growth rate of E. coli was greater in 10% sunflower oil emulsion than in the other emulsions. No physical changes were observed (Thomas et. al, 1983).

In research by Prachayio (2003) an oil-in-water emulsion was used as a model system to study the growth of E. coli O157:H7. The emulsions containing 0%, 5%, 20%, and 40% (w/v) hexadecane were fluid in texture with an average droplet size of 0.88. It was observed that there was a limitation of growth of the organism due to the effects of glucose diffusion. In addition it was noted that bacteria grown in emulsions had a higher frequency of curli production and were more heat resistant than cells grown in liquid media, suggesting that the bacterial physiology might vary in a heterogeneous system.

2.2.5 Effect of Preservatives

Many foodborne illnesses are commonly associated with the consumption of improperly cooked or stored food. A study has investigated the efficacy of dimethyl dicarbonate (DMDC), sulfur dioxide and sodium benzoate for eliminating E. coli. Food were treated with one of the following preservatives:

0.025% DMDC, 0.045% sodium benzoate (SB), 0.0046% sodium bisulfite (NAS), or a combination of NaS and SB and it was found that E. coli survived for 18 days in the control and that DMDC was the most effective safeguard at 4ºC and 10ºC, followed by the combination of NaS/SB, SB, and finally NaS. E. coli was more resistant to the preservatives at 4ºC than 25ºC. Even though DMDC was more effective at inactivating E. coli at 25ºC than the lower temperatures, this was no significant finding compared to the other preservatives (Fisher 1998).

2.2.6 Importance in Food

The reservoir of this pathogen appears to be mainly cattle and other ruminants such as camels. It is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products and raw milk. Faecal contamination of water and other foods, as well as cross-contamination during food preparation will also lead to infection. Examples of foods implicated in outbreaks include yogurt, cheese and milk (FSA 2012). Waterborne transmission has been reported, both from contaminated drinking water and from recreational waters (Public Health England, 2010).

Person-to-person contact is an important mode of transmission through the oral-faecal route. An asymptomatic carrier state has been reported, where individuals show no clinical signs of disease but are capable of infecting others (Jay 1996). The duration of excretion of EHEC is about one week or less in adults, but can be longer in children. Visiting farms and other venues where the

general public might come into direct contact with farm animals has also been identified as an important risk factor for EHEC infection (FSA 2014).

2.3 SALMONELLA SPP

Salmonella is a genus of rod-shaped, Gram negative non-spore-forming, predominantly motile bacteria around 0.7 to 1.5μm by 2 to 5 μm, and peritrichous flagella. They obtain their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes. Most species produce hydrogen sulfide which can readily be detected by growing them on media containing ferrous sulfate. Most isolates exist in two phases: a motile phase I and a nonmotile phase II. Salmonella is closely related to the Escherichia genus and is found worldwide in cold- and warm-blooded animals (including humans), and in the environment (Jay 1996).

2.3.1 Pathogenicity and Virulence

The organism enters the digestive tract and must be ingested in large numbers to cause disease in healthy adults. Gastric acidity is responsible for the destruction of the majority of ingested bacteria. Infection usually occurs as a result of ingestion of foods in which the bacteria are highly concentrated similar to the levels in a culture medium. However, infants and young children are much more susceptible to infection, easily achieved by ingesting a small number of bacteria. The infectious rate is also food dependent, as high fat food such as chocolate and cheese or acidic food can have a very low infectious dose. This appears to protect

the bacteria in their passage through the stomach and an infectious dose of about 100 bacteria has been reported in such incidents (PHE 2009).

Human salmonellosis comprises several clinical syndromes including enteric (typhoid) fever, localised enterocolitis and systemic infections by nontyphoid microorganisms. Clinical manifestations of enteric fever appear after a period of incubation ranging from 7 to 28 days and may include diarrhoea, prolonged and intermittent fever, abdominal pain and headaches. Human infections with non-typhoid salmonellae commonly result in enterocolitis that appears 8 to 72 h after ingestion of the invasive pathogen. Human infections with nontyphoid strains can also progress to systemic infections and result in various chronic conditions such as reactive arthritis, Reiter's syndrome and ankylosing spondylitis (European Commission 2003).

It has been shown that, in infants, the contamination could be through inhalation of bacteria-laden dust (Holliday 2003). After a short incubation period of a few hours to one day, the bacterium invades the intestinal lumen causing an intestinal inflammation with diarrhea that is often bloody. In infants, dehydration can cause a state of severe toxicosis (Holliday 2003).

2.3.2 Importance in Food

Salmonella infections are zoonotic and can be transferred between humans and nonhuman animals. Many infections are due to ingestion of contaminated food. A distinction is made between enteritis Salmonella and typhoid/paratyphoid

Salmonella such as Salmonella enterica subsp. enterica serovar Typhi, where the latter — because of special virulence factors and a capsule protein (virulence antigen) — can cause serious illness, Salmonella typhi is adapted to humans and does not occur in animals (Holliday 2003).

So far no outbreak has been reported of Salmonella spp in spreads. But as per EU Micro Criteria 2005, as a Food Business Operator (FBO) it must be ensured that any Ready To Eat (RTE) food products are free of the pathogen.

2.4 STAPHYLOCOCCUS AUREUS

Staphylococcus aureus is frequently part of the skin flora found in the nose and on skin, and in this manner about 20% of the human populations are long-term carriers of S. aureus. S. aureus is the most common species of staphylococci to cause staph infections in humans.

2.4.1 Pathogenicity and Virulence

Strains are responsible for food poisoning through the production of an enterotoxin and pathogenicity is also associated with coagulase positivity. S. aureus may occur as a commensal on skin, it also occurs in the nose frequently (in about a third of the population) and the throat less commonly. The occurrence of S. aureus under these circumstances does not always indicate infection and, therefore, does not always require treatment (indeed, treatment may be ineffective and recolonisation may occur). It can survive for hours to weeks, or even months, on dry environmental surfaces, depending on strain (Jay 1996).

A toxin dose of less than 1.0 microgram in contaminated food will produce symptoms of staphylococcal intoxication. This toxin level is reached when S. aureus populations exceed 100,000 per gram (PHE 2009). The Staph toxin is of main concern in the dairy industry in the cheese making factory as in a slow acidification process. Any vat of cheese, which has a low acidity within 6 hours of rennet addition could pose a hazard to health because of the possibility of the growth of Staphylococcus aureus that may further form toxin while growing.

2.4.2 Growth Characteristics

Staphylococci grow in as high as 80% milk fat, but 40% is the optimum (Voysey 2009). The tolerance of Staphylococcus aureus to high concentrations of sodium chloride in liquid medium has been reported (Jay 1996). It can grow at 37ºC in Tryptose Phosphate Broth with 0.85% sodium chloride. In contrast, solutions of sodium chloride in distilled water were injurious to staphylococci and killed most of these organisms in 1 h. Staphylococci were killed faster at 37ºC than at room temperature in a solution of 0.85% sodium chloride in water (Parfentjev 1964).

2.4.3 Importance in Food

Foods that are frequently incriminated in staphylococcal food poisoning include meat and meat products; poultry and egg products; salads such as egg, tuna, chicken, potato, and macaroni; bakery products such as cream-filled pastries, cream pies, and chocolate eclairs; sandwich fillings; and milk and dairy products. Foods that require considerable handling during preparation and that are kept at

slightly elevated temperatures after preparation are frequently involved in staphylococcal food poisoning (Jay 1996).

Staphylococci exist in air, dust, sewage, water, milk, and food or on food equipment, environmental surfaces, humans and animals. Humans and animals are the primary reservoirs. This incidence is even higher for those who associate with or who come in contact with sick individuals and hospital environments. Although food handlers are usually the main source of food contamination in food poisoning outbreaks, equipment and environmental surfaces can also be sources of contamination with S. aureus (FSA 2013). Human intoxication is caused by ingesting enterotoxins produced in food by some strains of S. aureus, usually because the food has not been kept hot enough (60°C, 140°F, or above) or cold enough (7.2°C, 45°F, or below) (Jay 1996).

2.5 BACILLUS SPP

Bacillus spp is a ubiquitous organism, producing spores, which can survive harsh environments. All dairy products are at risk from Bacillus spp in various ways due to the range of characteristics they comprise. Both types of housing strategy, conventional farming and organic farming can lead to contamination of raw milk and pelleted feed was found to contain a higher number of thermoduric microorganisms.

The genus Bacillus is a large and diverse group of bacteria belonging to the family Bacillaceae, Phylum Firmicutes. The species in this genus are aerobic or facultatively anaerobic, endospore forming, gram positive bacteria widely distributed in nature (Jay, 1996). Bacillus can be identified as rod-shaped cells (ca. 0.002 mm by 0.004 mm) that are able to propagate and the small (0.001 mm) oval shaped spores (Jay, 1996). Bacillus species are spore forming microorganisms that can grow over a wide range of temperature of 4 to 55°C, grows from at pH of 4.3 to 9.3 and requires a minimum water activity (aW) of 0.93 (Forsythe, 2000). Bacillus spp are thermoduric psychotrophs demonstrating how the organism is ubiquitous in nature and represents a substantial potential for spoilage of perishable milk products, which consequently has a significant influence on milk quality and shelf life (Meer et al., 1991).

The presence of B. cereus in pasteurised milk is a major concern for the dairy industry as the organism is associated with both spoilage and possesses pathogenic properties. B. cereus species are psychotrophic and are capable of growing under refrigeration, which magnifies the risk within the dairy industry. B. cereus spores are robust surviving pasteurisation temperatures and are also the most adhesive and hydrophobic among Bacillus spp, increasing the risk of biofilms formation.

B.cereus causes two types of food-borne illnesses. One type is characterized by nausea and vomiting with no abdominal cramps and has an incubation period of 1 to 6 hours. It resembles Staphylococcus aureus (staph) food poisoning in its

symptoms and incubation period. This is the "short-incubation" or emetic form of the disease and is due to a preformed toxin made during growth as with S. aureus. The second type is manifested primarily by abdominal cramps and diarrhoea following an incubation period of 8 to 16 hours. Diarrhoea may be a small volume or profuse and watery. This type is referred to as the "long-incubation" or diarrhoeal form of the disease and it resembles food poisoning caused by Clostridium perfringens. In either type, the illness usually lasts less than 24 hours after onset. In a few patients symptoms may last longer (Jay 1996, PHE 2003).

There are no specific requirements for Bacillus cereus and other species in foods under European Community (EC) legislation. EC legislation does require, however, that foodstuffs should not contain microorganisms or their toxins in quantities that present an unacceptable risk for human health. Both the UK Health Protection Agency (HPA) and the Food Safety Authority Ireland (FSAI) have published guidelines on acceptable levels of microorganisms in various ready-to-eat foods (see links below). These state that the acceptable level of Bacillus cereus and other pathogenic Bacillus species in these products is $\leq 10^4$ cfu/g.

2.5.1 Infective Dose

The presence of large numbers of B. cereus (greater than 10^6 organisms/g) in a food is indicative of active growth and proliferation of the organism and is consistent with a potential hazard to health (PHE 2009).

2.5.2 Effect of Salt Concentration

Sodium chloride, at concentrations of 2.0 and 4.0% in brain heart infusion (BHI) broth, had no effect on the thermotolerance of the strain. Changes in resistance to heat or refrigeration temperatures, as well as tolerance to NaCl, influences the ability of B. cereus to grow in minimally processed foods during distribution and storage (Mahakarnachankul 1999).

2.5.3 Growth Characteristics

Spores are more heat resistant in high fat or low water activity products (Jay 1996). The vegetative cells of Bacillus species are not notably resistant to commonly used preservatives, but the spores are much more difficult to destroy. The 'natural preservative' nisin, which prevents spore germination, has been shown to be effective at preventing the growth of Bacillus species in various food commodities (Jay 1996).

2.5.4 Outbreaks

Most outbreaks of Bacillus food poisoning are associated with the consumption of cooked food which has been cooled too slowly and/or incorrectly stored, providing conditions for the microorganism to increase to significant numbers (RASSF Portal 2014). The few outbreaks as per RASSF portal on Bacillus spp are mainly in cheese in Europe within 2010 and 2013. In 2014 there has been an incidence of B. subtilis in flavoured milk in Europe with a count of 300 cfu/ml. In

another incidence in UK, cream was withdrawn from the market due to B. cereus being present > 10^3 cfu.ml.

Bacillus cereus has been recognized as an agent of food poisoning since 1955. There are only a few outbreaks a year reported by CDC. Between 1972 and 1986, 52 outbreaks of food-borne disease associated with B. cereus were reported to the CDC (in 2003, there were two), but this is thought to represent only 2% of the total cases that have occurred during these periods. It is not a reportable disease, and usually goes undiagnosed.

2.5.5 Importance in Food

A wide variety of foods including meats, milk, vegetables, sauces, puddings, soups, casseroles and fish have been associated with the diarrhoeal type food poisoning. The vomiting-type outbreaks have generally been associated with rice products; however, other starchy foods such as potato, pasta and cheese products have also been implicated. Food mixtures such as pastries and salads have frequently been incriminated in food poisoning outbreaks.

2.6 YEAST AND MOULD

Yeasts are eukaryotic microorganisms in the kingdom of fungi, with 1,500 species currently described estimated to be only 1% of all fungal species. Most reproduce asexually and many do so via an asymmetric division process called budding. Yeasts are unicellular, although some species may become multicellular through

the formation of a string of connected budding cells known as pseudo hyphae. Yeast size can vary greatly depending on the species, typically measuring 3–4 µm in diameter, although some yeast can reach over 40 µm.

Moulds are a large and taxonomically diverse number of fungal species with hyphae. The networks of these tubular branching hyphae in moulds are called mycelium and are considered as one mould colony. There are thousands of known species of moulds which include opportunistic pathogens, saprotrophs, aquatic species, and thermophiles. Like all fungi, moulds derive energy not through photosynthesis but from the organic matter in which they live. Typically, moulds secrete hydrolytic enzymes, mainly from the hyphal tips. These enzymes degrade complex biopolymers such as starch, cellulose and lignin into simpler substances which can be absorbed by the hyphae. In this way, moulds play a major role in causing decomposition of organic material, enabling the recycling of nutrients throughout ecosystems. Many moulds also secrete mycotoxins which, together with hydrolytic enzymes, inhibit the growth of competing microorganisms (Jay 1996).

Moulds reproduce through small spores, which may contain a single nucleus or be multinucleate. Mould spores can be asexual or sexual; many species can produce both types. Mould spores may remain airborne indefinitely, may cling to clothing or fur, or may be able to survive extremes of temperature and pressure.

Although moulds grow on dead organic matter everywhere in nature, their presence is only visible to the unaided eye when mould colonies grow. A mould colony does not comprise discrete organisms, but an interconnected network of hyphae called a mycelium. Nutrients and in some cases organelles may be transported throughout the mycelium. In artificial environments like buildings, humidity and temperature are often stable enough to foster the growth of mould colonies, commonly seen as a downy or furry coating growing on food or other surfaces.

2.6.1 Infective Dose

The yeasts and moulds are widely dispersed, being found in a variety of locations and are virtually ubiquitous in any environment. Mycotoxins are poisonous chemical compounds produced by certain fungi found in food and animal feedstuffs such as grains and seeds. Mycotoxins are associated with diseased or mouldy crops, although the visible mould contamination can be superficial also in mould ripened food as stilton. Air, water, environment and the hospital can be a source of air borne infection on top of being food borne (Jay 1996).

Yeasts are very common in the environment, and are often isolated from sugar-rich material as skins of fruits and plants. They can also be associated contamination via soil along with being a natural skin and gut flora of mammals (Jay 1996). Infective dose for yeasts can be as little as 15 to 20 cells for example Candida spp, Saccharomyces cerevisiae in cheese, fruit and vegetables and

carbonated drinks (RASFF alert). For moulds it may vary from 10 to 100 organisms (PHE 2009).

2.6.2 Growth Characteristics

Yeasts are ubiquitous with the ability to survive and grow at low pH, low water activity, and in the presence of some common chemical preservatives, making them potent food spoilage organisms responsible for large economic losses of some food products. Low salt concentration has no effect on yeasts. But as yeast have a semi permeable membrane, high salt concentration will dehydrate the cells by osmosis. Salt stress is accompanied by an increase in the intracellular level of glycerol, free amino acids (notably proline and aliphatic amino acids), and Na+, as well as by changes in lipid and fatty acid composition (Mulet et al. 1999).

Moulds are slow growing aerobic organisms compared to bacteria and yeasts in normal conditions that are usually confined to the surfaces of foods, forming easily visible, often coloured colonies. Under unfavourable conditions for bacteria as in low aW or pH, moulds cause spoilage due to no competition (Jay 1996). Then the moulds can take over, forming hyphal mats or colonies on the surface of food products. Salt solutions will slow the growth rate of moulds as it dehydrates the cell by osmosis (Jay 1996).

Moulds can be xerophilic as well, where they are capable of growth below 0.85 aW as they have biochemical pathways that activate in these adverse environment

conditions, making them one of the biggest groups of spoilage microorganisms in processed and ambient storage food (Jay 1996).

Their membrane osmosensors contain glycerol that balances the osmotic pressure inside and outside the cell wall. Moderate xerophiles include species within Aspergillus, Penicillium and Eurotium spp. Extreme xerophiles compete poorly at high aw, because they require decreased aw for growth. Some xerophiles have a preference for salt or sugar substrates, whereas other species can be isolated from both jam and salterns. Xerophiles are widely spread on the fungal tree of life (Jay 1996). The yeast genus Zygosaccharomyces has had a long history as a spoilage yeast within the food industry with a wide range of pH (2.0 – 7.0) and aW (0.80 – 0.99) for growth. This is mainly due to the fact that these species can grow in the presence of high sucrose, ethanol, acetic acid, sorbic acid, benzoic acid, and sulphur dioxide representing some of the commonly used food preservation methods (Jay 1996). Yeasts are able to tolerate high ethanol concentrations (≥ 15% (v/v)). Z. bailii can ferment hexose sugars (e.g. glucose and fructose) and cause spoilage from an extremely low inoculum (e.g. one viable cell per package of any size). Hence RTE food with low pH (2.5 to 5.0) and aw (<0.85) are at risk of yeast spoilage.

2.7 AIMS AND OBJECTIVES

Spreads undergoe breakdown of the fat and components are produced which may give rise to changes in both the physical and organoleptic properties of the product. Fatty acids can be broken down further into smaller volatile molecules such as aldehydes and (methyl) ketones. These gave rise to cheese odours within the product that can be detected at low levels organoleptically and thus makes it undesirable to the consumers. The aim of the project was to look into identifying the source of spoilage that eventually induce a specific cheese smell in the product, improve processing condition to make a better quality product and to benchmark it against the competitors.

The objectives were to characterise the microorganisms and establish the root cause of the issue. The identified strains would be used as a library of strains to challenge test the ingredients, other spreads and different processing conditions. The product structure in terms of rheology will also be analysed to measure the effect of spoilage. Figure 2.2 outlines the study flow diagram.

Listeria spp is one of the main dairy environment contaminants found across the world (RASFF alert) and results in various recalls, withdrawal and outbreak incidences. As a part of the study the growth pattern of the organism was validated under various processing conditions, environment and formulation to develop a typical dairy growth model.

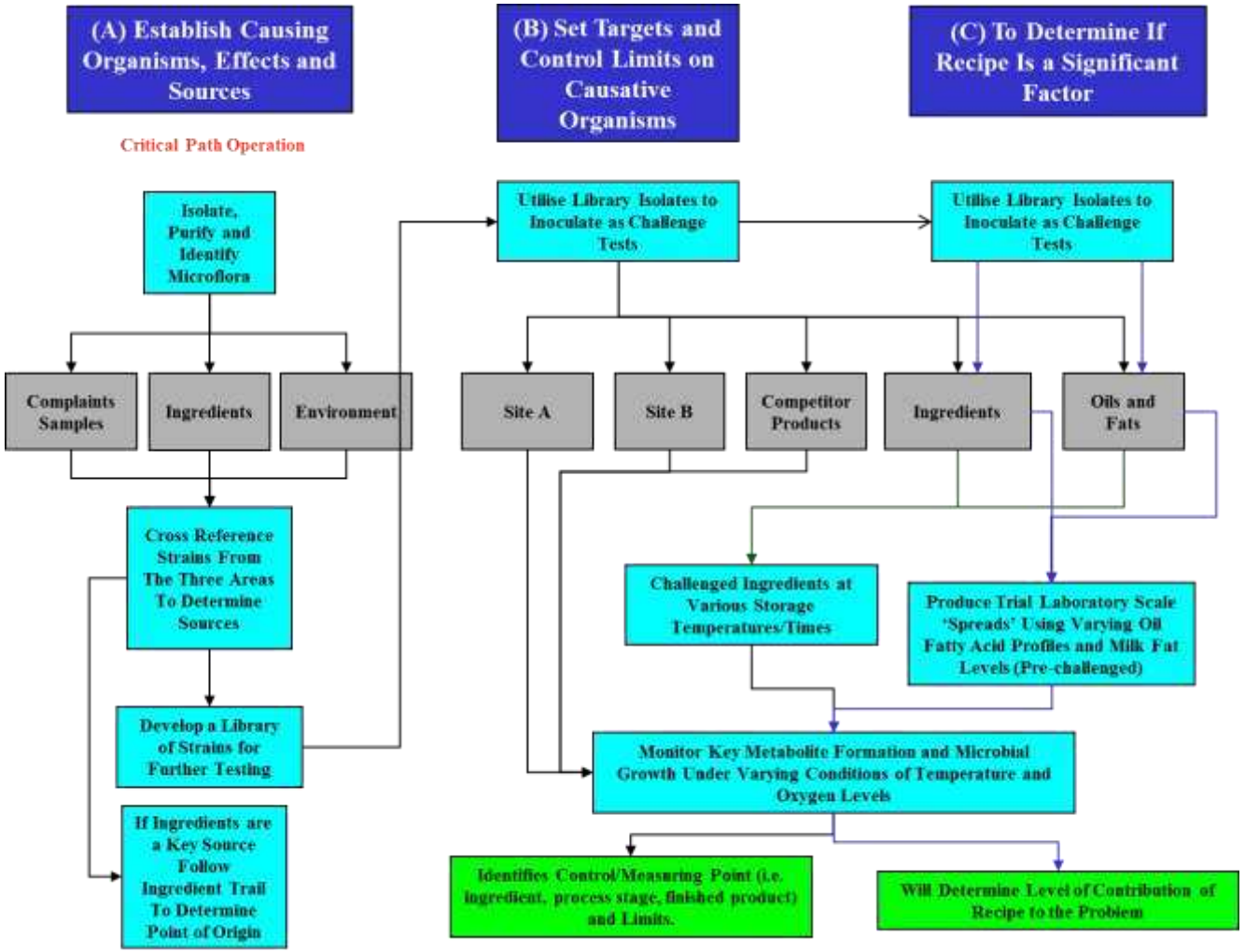


Figure 2.2 Summary of the study outline

CHAPTER 3 METHODS AND MATERIALS

3.1 MICROBIOLOGY SAMPLE PREPARATION AND ANALYSIS

All the microbiology methods used for this study were internationally recognised ISO methods published by International organisation of Standardisation for microbiology. Some methods also followed specific British Standards (BS). The sample preparations were as per ISO 17025 and refined for spread sampling.

3.1.1 Product and ingredient preparation

The finished spread samples and its ingredients such as cream, buttermilk, skimmed milk and oil blends were melted in a sterile container in a water bath at 45 ± 1°C. For general microbiology testing the samples were well mixed and 10 ml was placed into a 250ml Duran bottle containing 90 ml Maximum Recovery Diluent MRD (Appendix 10.3.1) at 45 ± 1°C and mixed thoroughly. Liquid milk was used directly for analysis at 20ºC. For dilutions 1ml milk was mixed directly into 9 ml of MRD.

3.1.2 Non-selective methods for isolation of bacterial strains

Total Viable Count (TVC) gives a general indication of the bacterial load in the product. The pour plate technique was used for total viable counts on non-selective media and for more specific groups of microorganisms grown on selective media. One ml of the sample was mixed with an approximate 20 ml of

the medium at 45ºC in a petri dish and mixed thoroughly by turning the plate 5 times clockwise and anticlock wise (BS 4285: Section 1.1 1991). The plate was allowed to set prior to incubation at 30ºC for 48 h on the non-selective medium Milk Plate Count Agar MPCA (Appendix 10.3.1.2) after which typical colonies were counted (BS 4285: Section 1.1 1991) of the similar characteristics in shape, size and form.

3.1.2.1 Colony morphology study

Colony morphology was used for presumptive identification as bacteria show characteristic type of growth on solid medium. The colonies were observed after being incubated on MPCA for 48 h at 30ºC of their varied forms (circular, irregular, filamentous, rhizoid etc.), shape, texture, colour, sizes, elevation (flat, raised, convex, etc.), translucency (transparent, opaque, translucent) and margin (entire, undulate, lobate etc.). Based on similar characteristics the bacterial isolates were grouped for further selective isolation and identification.

3.1.2. 2 Gram staining

The Gram stain technique is a differential double stain that divides bacteria into two taxonomic groups. The Gram stain forms the basis of preliminary identification of bacterial cell morphology. An 18-24 h old bacterial culture was used to prepare a smear. A loopful of (Maximum Recovery Diluent MRD Appendix 10.3.1) was transferred to the surface of a clean glass slide. One colony was transferred into the diluent, emulsified and spread out as a thin film that was

then allowed to dry and heat fixed briefly by passing through the Bunsen burner three to five times. The smear was then stained with crystal violet (Prolab, UK) for one minute, washed off with water, treated with mordant, Lugol's iodine solution (Prolab, UK) for 30 seconds and decolourised with ethanol for one minute. After each stage the excess was washed off with tap water. Finally the slide was counter stained using carbol fuschin (Prolab, UK) for 30 seconds and washed again with tap water and the slide was allowed to dry for approximately 30 minutes before examining under an oil immersion microscope at a X 1000 magnification.

3.1.2.3 Wet mount technique

The wet mount technique was used to observe the morphology of Yeast and Mould (Y&M). A single isolated Y&M colony was transferred onto a glass slide and using a Pasteur pipette, a drop of sterile water was placed on the sample. One end of a cover slip was gently placed on the slide and slowly lowered to help to prevent air bubbles from getting trapped under the cover slip. The Y&M were observed under microscopy at 1000 x magnification

3.1.2.4 Catalase test

For the catalase test 0.1ml or one drop of hydrogen peroxide (30% v/v, Fischer Scientific, UK) was added into a petri dish and mixed with two to three bacterial colonies (24 hrs old maximum) and examined for the production of gas bubbles.

Vigorous bubbling was taken as a positive test and no bubbling was considered negative. A hand lens was used for very slight catalase production. Pediococcus pentosaceus and Pseudomonas fluorescens SM06 (University of Nottingham, Microbiology and Food Safety laboratory culture collection) were used as the negative and positive controls respectively.

3.3.1.4 Oxidase test

The oxidase test reagent contains tetramethyl-p-phenylenediamine dihydrochloride, which rapidly converts to oxidised (purple coloured) products by oxidase positive species containing Cytochrome C. A loopful of the reagent was placed on a filter paper to soak. One single isolated colony was picked and transferred onto the paper using plastic loops or cocktail sticks. The contact time between the cells and test reagent was 30 seconds. Formation of a purple colour denoted a positive test. Pediococcus pentosaceus and Pseudomonas fluorescens SM06 were used as the negative and positive controls respectively.

The reagent used in the oxidase test may autooxidise; hence fresh reagents were used all the time. A false positive result may be achieved if using nickel, steel or other loops.

3.1.3 Selective medium for isolating pure cultures

The spread plate method was used for selective isolation. Sample (0.1 ml) was pipetted onto the surface of a set agar in a petri dish and spread evenly over the whole surface of the agar using a sterile spreader. The inoculated plates were

incubated as appropriate for the medium after which typical colonies were counted (BS 4285: Section 1.1 1991). Each sample was plated out in duplicate and colonies were counted in the range of 30 to 300 cfu and expressed as log cfu / ml or /g.

3.1.3.1 Isolation and identification of Enterobacteriaceae family

Enterobacteriaceae were detected using pour plates with a selective medium Violet Red Bile Green VRBG Agar (Appendix 10.3.1.3). Approximately 15 ml of agar was poured onto 9 cm plates and mixed thoroughly or for 14 cm plates approximately 40ml of agar was used and mixed thoroughly. It is important to ensure even dispersion of the sample within the medium by mixing with rapid circular and to and fro movements. If any large bubbles were formed in the agar at this stage they were burst with a sterilized straight wire before the agar sets. Once the plates are set, overlay each plate with about 10ml of agar for 9cm plates and at least 20ml of agar for 14cm plates. Ensure a total covering and allowed to set. The overlay restricts motile organisms and to produce partially anaerobic conditions. The plates were incubated at 24 ± 2 h at 37 ± 1°C and any purple colonies were counted (ISO 21528-2: 2004).

3.1.3.2 E.coli

The pour plate technique was used for inoculating 1 ml of the samples into the TBX medium (Appendix 10.3.1.4) and plates were incubated at 37 $\pm$ 1°C for four

hrs and then transferred for 18 - 24 h. incubation at the selective temperature of 44 ± 0.5° C. Plates containing blue /green colonies were counted as presumptive E. coli (ISO 16649-2:2001).

3.1.3.3 Isolation and identification of Salmonella spp

To isolate Salmonella spp 25 g of the prepared samples of spread were diluted in one to nine with and incubated at 37°C ± 1°C for 18 ± 2 h. One ml of the pre-enriched broth was inoculated into selective enrichment broth 10 ± 0.2 ml Muller-Kauffmann tetrathionate-novobiocin broth MKTTn (Appendix 10.3.1.6) and incubated for 24 ± 3 h. at 37 ± 1°C. The selective broth will inhibit most other microorganisms and allow any Salmonella spp present to dominate. After incubation a loopful was streaked onto selective Xylose Lysine Deoxycholate XLD (Appendix 10.3.1.7) and incubated at 37 ± l° C for 24 ± 3 h and examined for typical or non-typical Salmonella (BS 6785:2007), i.e., respectively red colonies with black centres, red colonies, yellow colonies with black centres, or yellow colonies with a narrow clear margin. Non-typical colonies are regarded as a negative result. Salmonella Senftenberg will appear as red colonies on the medium that is hydrogen sulphite negative. But as it is mainly predominantly found in poultry, red colonies were discounted.

3.1.3.4 Isolation and identification of Bacillus spp

Bacillus spp were isolated by heating the samples at 80 ± 1°C for ten minutes in a stirred water bath. Colonies from surviving spores were then enumerated using a non-selective medium MPCA (Appendix 10.3.1) using the spread plate technique (Section 3.1.1). The set plate was incubated at 30 ± 1°C for 72 ± 4 h, Polymyxin Pyruvate Egg Yolk Mannitol Blue Agar PEMBA (Appendix 10.3.1.8) contains bromothymol blue pH indicator that gives clear visualisation of alkaline mannitol nonfermenting colonies and egg yolk precipitation indicative of B. cereus. The selectivity is provided by the polymyxin B supplement (X193) and provides excellent results for the majority of sample types. Typical colonies are at least 2-3mm in diameter with serrated (scalloped) edges turquoise or peacock blue in colour which may or may not have a precipitate of the same colour round the colony. Other species of Bacillus colonies have specific characteristics as in Table 3.1. Yellow colonies are formed by mannitol-fermenting Bacillus subtilis and B licheniformis.

Table 3.1 Bacillus spp growth characteristics on PEMBA (Bergey's 2010)

GROWTH CHARACTERISTICS		
Organism	Colony size (mm)	Colour
B. cereus	3.0-4.0	Blue white halo
B. subtilis	2.0-3.0	Yellow
B. licheniformis	2.0	Yellow

3.1.3.5 Isolation and identification of Staphylococcus spp

Mannitol Salt Agar (MSA) (Oxoid CM0085) (Appendix 10.3.1.9) is a selective medium for the isolation of presumptive pathogenic staphylococci. Most other bacteria are inhibited, with the exception of a few halophilic species was spread plated with 0.1ml samples and then incubated for 36 h at 35°C (BS 4285:1989). Presumptive coagulase-positive staphylococci produce colonies with bright yellow zones whilst coagulase-negative staphylococci are surrounded by a red or purple zone. Staphylococcus and Micrococcus spp can also be detected by the use of potassium tellurite and lithium chloride as selective agents. The diagnostic system is based on tellurite reduction (blackening) and the activity of protease (clearing), lipase (white halo) and lecithinase (opalescent ring). 0.1ml of inoculum was spread onto Baird Parker agar (BPA) (Appendix 10.3.1.10) and incubated at 37°C ± 1°C for 24 ± 2 h. Typical colonies are black, shiny, and convex and 1 to 3 mm in diameter surrounded by a clear zone or halo, which may be partially opaque and may also contain an opalescent ring and reported as presumptive S. aureus. Atypical colonies may be less black with a rough dry texture or may be black and shiny without any halos and not frequently associated with dairy products, but can occur in other food types. Suspect colonies were picked off into brain heart infusion broth (BHI) (brain infusion solids 12.5 g/l, beef heart infusion solids 5.0 g/l, proteose peptone 10.0 g/l, glucose 2.0 g/l, sodium chloride 5.0 g/l, disodium phosphate 2.5 g/l, pH 7.4 ± 0.2) (Oxoid CM 1135) and incubated at 37 ±1°C for 18 to 24 h to promote growth and coagulase production. An aliquot of broth culture was used for the coagulase test. If the coagulase test yielded a positive result, the original colony was assumed to be S.aureus. The Micrococcus

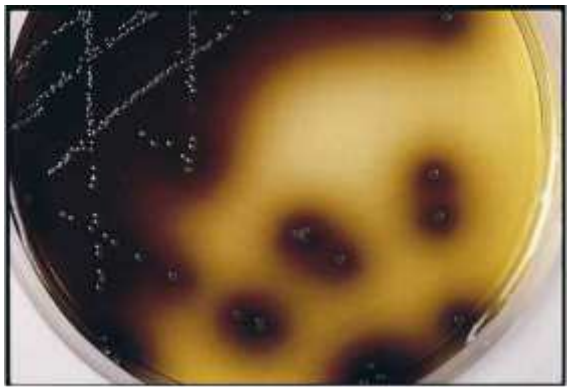
spp colonies are usually of smaller size and under the optical microscope at x1000 magnification are seen as shorter chains of two to four cells.

3.1.3.6 Isolation and identification of Listeria spp

For isolating and identifying Listeria spp using the standard ISO method, a 25 g sample was emulsified with 225ml Listeria enrichment broth (Appendix 10.3.1.11) and enriched for two days at 30 ± 1°C. A loopful was then streaked onto a Listeria selective agar Oxford (Appendix 10.3.1.12). The plates were incubated at 30 ± 1° C for 48 ± 4 h and examined for typical colonies. On Oxford agar typical colonies after 24 h are grey/black, but can sometimes appear greyish green, 1mm diameter, and surrounded by a black halo. At 48h colonies on Oxford agar are 2-3 mm in diameter, and black with a black halo and sunken center (BS EN ISO 11290-1:1997, BS 5763-18: 1997).

Listeria spp were also isolated (ISO 10560:1993) using a basic enrichment culture method. Buffered Listeria Enrichment Broth was inoculated with the test portion and incubated at 30º ± 1°C for 48 ± 4 h. Listeria Selective Agar. LSA was inoculated from the Buffered Listeria Enrichment Broth, and incubated at 30ºC and examined 24 ± 4 hrs. and 48 ± 4 h. for the presence of typical Listeria colonies and further confirmed by Gram stain, oxidase and catalase test.

Figure 3.1 Typical Listeria spp colonies on LSA agar



For Listeria spp identification a fully automated system VIDAS (Biomeriuex VIDUS Listeria Duo LDUO 13282 -2007 / 7 manufacturer's manual) was also used.

For Listeria spp enumeration test was also carried out for challenge test efficiency evaluation, a test sample was enriched in Listeria Enrichment Broth for two days at 30 ± 1°C. A loopful was then returned and streaked onto a Listeria Selective Agar (Oxford) plate. Plates were incubated at 30 ± 1°C for 48 ± 4 h and examined and counted for typical colonies as per ISO 10560:1993.

On Oxford and PALCAM, typical colonies after 24h are grey/black, but can sometimes appear greyish green, 1mm diameter, and surrounded by a black halo. At 48h colonies on Oxford agar are 2-3 mm in diameter, and black with a black halo and sunken Centre. On PALCAM colonies may appear grey-green with a black sunken center and black halo against a cherry-red background If alternative agars are used they should be incubated according to the manufacturer's

recommendations, and typical colonies should be identified according to manufacturer's instructions.

3.1.3.7 Isolation and identification of Pseudomonas spp

To isolate Pseudomonas spp 0.1 ml of prepared samples was spread onto the surface of a prepared set agar plate containing a selective medium CFC (Appendix 10.3.1.13). Typical Pseudomonas colonies appear as off-white in colour, sometimes with yellow or greenish tinges, and often associated with strong "fruity" odours. Plates with 15-150 typical colonies were counted (ISO 13720:1995).

3.1.3.8 Isolation and identification of proteolytic microorganisms

Proteolytic microbes were isolated on a Caseinate Medium was inoculated with 0.1 ml of prepared samples by the spread plate method and incubated at 30± 1° C. The plates were examined after 3 days for mesophiles and at 5°C for 14 days for psychrophiles. The proteolytic microorganisms formed a precipitate of paracadein in the transparent medium by utilizing the caseinates (CCFRA 2003).

3.1.3.9 Isolation and identification of lipolytic microorganisms

Lipolytic bacteria isolation was performed by spreading 0.1 ml of prepared samples on to Tributyrin Agar (TBA) (Appendix 10.3.1.15) and incubated at 30 ±

1°C for 72 ± 4 h. Typical creamy circular colonies in an otherwise opaque medium (BS 4287:1989) surrounded by a clear zone >1mm were scored as lipolytic.

3.1.3.10 Isolation and identification of Lactic acid bacteria

MRSA (de Man, Rogosa, Sharpe Agar) (Appendix 10.3.1.16) plates were spread with 0.1ml samples and incubated at 30°C ± 1°C for 72 ± 4 h. All the colonies were counted and confirmed by performing a Gram-Stain and catalase test (ISO 15214:1998). Many lactobacilli are Gram variable and begin to appear Gram negative after 16-18 h; therefore the Gram stain should be done as soon as possible. The bacteria are Gram positive, catalase-negative cocci or non-sporing rods.

3.1.3.11 Isolation and identification of yeasts and moulds

Czapek Dox liquid medium (same formulation as Czapek Dox Agar (modified) Appendix 10.3.1.17 but lacking the agar) was used for an enrichment stage prior to plating it out on the agar plate. The broth was incubated at 25 ± 1°C for 24 h in a ratio of 10:1 ml for the broth and sample respectively. After the pre-incubation stage, 0.1 ml of the enriched sample was spread plate onto pre-prepared Czapek Dox medium and incubated at 25 ± 1°C for five days and examined for the presence of moulds.

For yeasts Oxytetracyline Glucose Yeast Extract Agar OGYEA, Oxoid CM0545 containing yeast extract 5.0 g/l, glucose 20.0 g/l, agar 12.0 g/l and pH 7.6 ± 0.2; one vial of Oxytetracyline selective supplement Oxoid SR0073 was added per 500 ml of medium. Selective agar was used as it inhibits the bacterial growth. The medium was inoculated with 0.1 ml of prepared samples via the spread plate technique and incubated at 25 ± 1°C for five days and examined for the presence of yeasts. (ISO 21527-2:2008).

3.1.4 Environment Sampling

3.1.4.1 Air sampling

The factory air was microbiologically analysed using the pre-poured selective media PEMBA, MSA, Czapek Dox and OGYEA. The plates were exposed for a minimum of two h to capture the aerosols in the factory. An air sampler SAS Super 100 Digital microbial Sampler (calibrated externally annually, part of Good Laboratory Practice) was also used to draw directly 1000l of air in approximately 5 minutes. The plates were incubated as appropriate to the organism being isolated.

3.1.4.2 Potable Water Test

Potable water was also screened for microbial load, as it is an ingredient in the product. Water (1ml taken from a 100ml sample) was directly sampled using a pour plate method and incubated appropriate to the medium used.

3.1.4.3 Packaging Sampling

Packaging or containers to be rinsed were aseptically closed with either lid or with an alternative sterile closure. MRD (Appendix 10.3.1) was poured at a volume of 50 ml into the container and re-fitted the lid or closure. The diluent was then rotated and inverted to ensure that the diluent covers the entire internal surface and this process was repeated 10 times. Afterwards the diluent was returned to a sterile container and tested as per requirements within 6 hours stored under refrigeration conditions.

Results Calculation was performed as per below:

Count / ml obtained x total volume of diluent used

3.1.4.4 Personnel Screening

The random swabbing of hands / gloves should take place for staff directly handling food, after hand washing and / or on line. The hands are swabbed for Enterobacteriaceae and Staphylococcus spp. Commercially available microbiological swabs are used that are available ready-made and comes in a neutralizing buffer to swab the entire surface area of the inside and outside of the hand including the skin in-between each fingers (Figure 3.2). The swabs are tested as method for each organism in section 3.1.3.

Figure 3.2 Hand swabbing procedure

3.1.5 Analytical profile index (API) identification

The following analytical profile index (API) identification kits were used to identify some bacterial species in Section 2.1.3. This also helped in establishing the phenotypic similarities in the strains identified through selective medium microbiology and 16S rDNA sequence analysis. The tests were carried out using manufacturer's (BioMerieux) instructions for each test kit.

3.1.5.1 API 50 CHB

API 50 CHB (bioMerieux, Inc.) was used for identifying Gram positive catalase producing sporing rod isolates according to the manufacturer's instructions. It is a ready-to-use kit that allows the fermentation of 49 carbohydrates on the API strip.

A 24-h bacterial culture was taken from PEMBA and re-streaked onto a non-selective agar MPCA at 30 ± 1°C for 24 ± 2 h. After 24 h the pure isolates were

streaked in Nutrient Broth and incubated again at 30º C for 24 h. Each tube of the strip was then inoculated with the suspension and incubated overnight at 55°C. During incubation, carbohydrates fermented to acids produce a decrease in the pH, detected by a change in colour of the indicator phenol red in the medium to yellow. The results were read by comparing with the interpretation Table supplied by the manufacturer BioMerieux and the data were put into the computer software for species identification (API Identification software version 3.2.2). The apiweb software available online at the company's website (https://apiweb.biomerieux.com).

3.1.5.2 API STAPH

API STAPH (bioMerieux, Inc.) is an identification system for the genera Staphylococcus, Kocuria and Micrococcus, using standardized and miniaturized biochemical tests with a specially adapted database according to the manufacturer's instructions. API STAPH consists of a strip containing dehydrated test substrates in individual microtubes. The tests were reconstituted by adding to each tube an aliquot of API STAPH Medium that had been inoculated with the strain to be studied. A 24-h bacterial culture was taken from the selective plate and re-streaked onto a non-selective agar MPCA at 30 ± 1°C for 24 ± 2 h. Each tube of the strip was then inoculated with the suspension and incubated for 18-24 h at 35-37°C after which the results were read and interpreted according to the manufacturer's instruction. The identification was facilitated by the use of the API STAPH Analytical Profile Index or the identification software. The results were read by comparing with the interpretation Table supplied by the manufacturer

BioMerieux and the data were put into the computer software for species identification (API Identification software version 3.2.2).

3.1.6 MOLECULAR MICROBIOLOGY ANALYSIS

3.1.6.1 DNA extraction

Bacterial strains were grown (spread plate) on MPCA incubated overnight at 30 ± 1°C. DNA was extracted from a single isolated colony by adding 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and heating in a PCR block at 95º C for five minutes. The samples were amplified in a programmable thermocycler (Techne). Amplification conditions for plantaricin EF operon were as follows: initial denaturation at 94°C, 5 min; 30 cycles of 94°C, 1 min; 51°C, 40 s; 72°C, 3 min; and 72°C for 10 min. The extracted DNA was used directly as a template for PCR or stored at -20^{0}C for future use.

From the yeast and mould isolates pure cultures were grown on OGYEA at 25°C for 48 hrs. Two to three colonies were suspended in a sterile PCR tube (0.5ml Bio-Rad) containing 50 µl of extraction buffer (0.02M NaOH, 0.01% N-Lauroylsarcosine) using a sterile pipette tip. The cell suspension was heated at 99°C for 10 minutes in a PCR thermal cycler (TC-312; Techne) and then centrifuged at 16000-x g for 5 minutes (Biofuge Pico; Heraeus) in order to remove the non-soluble cellular material. The supernatant (35 µl) was transferred to a sterile Eppendorf tube. The extracts were used directly as a template for PCR or stored at -20°C for future use.

Table 3.2 Primer details for 16S rDNAPCR and 26S rDNAPCR amplification

PCR Type	Primer Name	Primer Sequence
16S PCR*	V3F	5'CCTACGGGAGGCAGCAG 3'
	V3R	5' ATTACCGCGGCTGCTGCTCG 3'
26S PCR**	NL1	5'GCCATATCAATAAGCGGAGGAAAAG 3'
	LS2	5' ATTCCCAAACAACTCGACTC 3'

*Chakravorty et.al. 2007

** Cocolin et al. 2002

3.1.6.2 Amplification of the Ribosomal DNA regions

For bacterial isolates the V3F and the reverse V3R (Chakravorty et.al, 2007) set of primers were used for amplifying the V3 variable region of 16S rDNA. Each 50 µl of mixture contained 25 µl of Taq polymerase, 1 µl of template DNA, each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 100 ppm and 23.8 µl of deionised water. Amplifications were performed in a TC-312 Techne thermal cycler. Template DNA was initially denatured for 5 min at 95°C followed by 30 cycles of 95°C for 1 min, 52°C for 45 s and 72°C for 1 min, completed with a final extension step of 72°C for 7 minutes.

For Y&M isolates the NL1 (5'CCATATCAATAAGCGGAGGAAAAG 3') and the reverse LS2 (5' ATTCCCAAACAACTCGACTC 3') set of primers were used for amplifying variable regions of 26S rDNA (Cocolin et al., 2000). Each 50 µl of

mixture contained 20 ng of template DNA (4.1.2.2), each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1.5 mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl and 1.5 U of Taq polymerase (Abgene). Amplifications were performed in a TC-312 Techne thermal cycler. Template DNA was initially denatured for 5 min at 95°C followed by 30 cycles of 95°C for 1 min, 52°C for 45 s and 72°C for 1 min, completed with a final extension step of 72°C for 7 min.

3.1.6.3 DNA extraction and sequencing

Amplified DNA (25 µl of PCR product mixed with 2 µl of 6X loading dye (Promega, UK)) from 16S and 26S PCRs were run in a 1% agarose (Melfod) gel containing ethidium bromide (0.2 µg/ml) and TAE running buffer (40 mmol 1^{-1} Tris base pH 8.0, 20 mmol 1^{-1} glacial acetic acid, 1 mmol 1^{-1} EDTA) for two hrs. at 70 volts. Gels were visualized in a UV transilluminator (Bio-Rad) and images were recorded using Quantity one Gel Doc software version 4.6.3 (Bio-Rad, USA).

The PCR bands containing the DNA were cut out from the agarose gel with a sterile blade and placed into sterile eppendorf tubes and either stored at –20ºC for future use or purified directly. For DNA purification Zymoclean Gel DNA Recovery Kit (Zymo Research D4002, USA) was used and manufacturer's instructions were followed. ADB buffer (D4001 USA, 500 µl) was added to the sample and incubated at 55ºC for a minimum of three hrs but usually overnight. The clarified solution was then transferred into a Zymo-spin column with a

collection tube and centrifuged for 30 seconds at 13,000 rpm. The DNA wash buffer (D4003, USA, 200 µl) was added to the column and centrifuged as before. Finally 10 µl of purified DNA was eluted and send for sequencing to MWG Eurofins (Ebersberg, Germany).

To determine the closest known relatives partial sequences obtained were searched in public data libraries using BLAST search tools.

3.2 CHEMICAL ANALYSIS

The test methods used for chemical analysis in this study were all industry standard method for spreads and ISO approved. In-house test method has been developed for the chemistry abuse testing that has been validated over the years. All the equipment and apparatus used in this section were calibrated, maintained and reliability ensured as per ISO 17025 (2005).

3.2.1 General chemical analysis

General chemical testing such as pH, salt, moisture and water activity was carried out in spreads.

3.2.1.1 Rheology study

Spread samples were allowed to equilibrate in either a water bath at 20ºC or in a storage area at room temperature for at least 1 h, preferably overnight. The Stevens-LFRA texture analyser was used to measure the load required to force a

plastic cone (10 mm width at the widest end and 2 mm at the narrow end) into the surface of a product at a constant speed up to a predefined depth. The parameters of the machine were set as distance 0.7 mm and speed 1 mm/sec. The load is proportional to the firmness of the product. The rheology results are quoted as 'g' load.

3.2.1.2 Free fatty acid (FFA) /methyl ketone analysis

Spread samples were warmed in a water bath or incubator at 35°C until softened. The warmed samples were mixed thoroughly and 10g were placed into a 40ml vial called the Monotrap RCC18 (Hichrom part no. 1050-72201) and incubated at 60°C for 3 h to liberate the volatile components and subsequently adsorbed onto the Monotrap that was transferred to the Autosampler vial insert. 1-1.2ml dichloromethane (Oxoid) was placed into a 2ml autosampler vial to conduct the ultrasonic waves into the insert into which the Monotrap was placed. An insert was then placed into the 2mls vial containing the 1-1.2 ml dichloromethane. The Monotrap then was placed into the insert and 200µl dichloromethane (chromatography solvent grade i.e., Sigma Chromasolv) was added. The vial was then sealed with a screw cap with integrated septum, and ultrasonicated for 4-6 minutes at ambient temperature. It was then placed into a Gas Chromatography (Back injector Solvent A dichloromethane, injector volume is 1µl, heater at 280°C, Pressure 13.518 psi, Column Pressure = 13.518psi. Flame Ionisation Detector (FID) Heater at 280°C with H2 flow = 40ml/min, Air Flow = 450ml/min, Makeup flow (N2) = 30ml/min and back signal FID (20Hz/0.01min) with 200µl

dichloromethane and placed in an ultrasonic bath and agitated for four to six minutes. The volatile components were extracted into the solvent via ultrasonic agitation before their concentration was measured against a prepared standard using capillary Gas Chromatography (Agilent 5975 Mass spectrometer and Marks International Series 2 Unity thermal desorption unit). This method allowedsimultaneous determination of methyl ketones with a carbon chain length of C4 - C13.

After 4 hrs of analysis per sample the generated chromatograms were assessed to identify the peaks of the desired methyl ketones. The height of the peaks generated from the analysis are related to the concentration of the presence of the MK and expressed as the fraction of the aliquot of sample taken for analysis.

3.2.1.3 pH

A HANNAH pH meter was used with temperature compensation and capable of being read to 0.01pH unit that has a glass electrode as a reference electrode. Reagents buffer solutions pH 4.0, 7.0 and 10.0 (Oxoid) were used to calibrate the instrument daily.

The spread samples for analysis were prepared by melting 200g at 65º C. The water phase of the sample (the separated lower portion of the oil) was carefully drawn off from the completely melted sample using a disposable pipette or similar and collected in a suitable container. This was thoroughly mixed and equal amounts were transferred to each of two centrifuge tubes and transferred to an ice-bath and submerged completely. After 20 to 30 minutes when the fat had

solidified, the sample was removed and the separated water phase was collected into another container and the pH was measured using the electrodes. The value was read to the nearest 0.01 pH unit(BS5086: Part 7:1991).

3.2.1.4 Salt

The spread sample was softened in a water bath at 35º C, until the sample was less fluid and easily stirred. A portion (1.8g - 2.2g) of well-mixed sample was mixed with 10ml of distilled water, 25ml of silver nitrate solution (Oxoid) (14.53g/l $AgNO_3$ stored in a dark bottle) and warmed on a hot plate at 70°C-80°C until a smooth well mixed sample was obtained. Nitric acid (10ml) was gently boiled and added to the mixture. The sample was then removed from the hot plate and allowed to cool. Ammonium iron (III) sulphate (2ml; Oxoid) indicator solution and 50ml of distilled water was mixed thoroughly and immediately titrated with the potassium thiocyanate solution (IRRITANT -50g/l K_2CrO_4 BDH AnalaR stored in dropping bottle) (Oxoid) until the solution in the flask showed an orange tint, which persisted for about 15 seconds. The burette reading was recorded to the nearest 0.05 ml. The percentage (%) of salt was calculated as: $NaCl = 0.292 (V1 - V_2)$ where V_1 is the blank titration and V_2 is the sample titration (ISO 1738:1980).

3.2.1.5 Moisture

Moisture levels were evaluated on the basis of changes in the water content after evaporation. On exposure to microwave radiation, water molecules in the product absorb energy and are heated and driven off through evaporation. Samples are

weighed before and after microwave irradiation, allowing the moisture loss to be calculated.

Filter papers (3M) were labelled and weighed (W1) and 3-4 g of the prepared sample was placed in the center of the filter paper. Another filter paper was used to cover the sample and weighed (W2) again. Afterwards the sample was pressed between the filter papers and each "sandwich" was placed into the microwave (800 watts) for 2-3 minutes. With the power setting at 100% the sample was heated for 2 minutes and then removed, allowed to cool on the bench for 3 minutes then re-weighed (W3).

$$\% \text{ Moisture} = \frac{[W2 - W3]}{[W2 - W1]} \times 100$$

This method is an in-house method validated against BS 5086: Part 2: 1995.

3.2.1.6 Droplet Size

A firm emulsion stored at 5ºC was carefully extracted using the wide end of a Pasteur pipette by inserting at least 1 cm into the emulsion / spread. The pipette end was carefully wiped clean and transferred into a dry test tube with the sample side facing downwards and secured tightly to prevent any movement and the top of the glass tube was sealed with paraffin film (Lab Scientific LTD). The sample was cooled in a coolant waterbath at 5ºC for 30 minutes.

The droplet size was then measured by using a Pulse Field Gradient Nuclear Magnetic Resonance using Droplet software V1 rev 2.

3.2.1.7 Water activity

To measure the water activity of the product a 10g sample was taken into an airtight container. A water activity meter (Hygrotec Labo 47), and a water-jacketed measurement chamber with a circulating water bath was used for the experiment. The water bath temperature was set at 20 ±0.5°C and allowed to equilibrate. A lens paper (3M) was used to remove any condensate. The prepared sample was placed within the measurement container ensuring the measurement head and chamber were lined up by re-tightening the knurled knob. The sample was allowed to equilibrate for 1 h and the % ERH (equilibrium relative humidity) reading was noted. The results were expressed to the nearest 0.1, as the %ERH stating the temperature of measurement.

3.2.1.8 Spoilage rate at various levels within a spread

A sample tub of spread was gently divided into 3 Sections (top, middle and bottom) aseptically by a sterile spatula. Each Section was then analysed for the presence of Free Fatty Acids / Methyl Ketones by a GC-MS method as per Section 3.2.1.2.

3.2.1.9 Sensory test for spreads

The tested sample should be at room temperature at 20ºC. The sample should be visually analysed and smelt prior to tasting and noted for any off smell or taints. The sensory test involving an aroma and taste is based on an in-house method. A triangle test was taken place for 10 trained sensory assessors for spread. For each test sample two other standard samples was also used to evaluate the degree of difference between each samples.

3.3 CHALLENGE TEST

The challenge tests in this section was developed in-house based on the product matrix and suitability of the assessments.

3.3.1 Single Purified Microbial Strain Preparation

All the isolated and identified bacteria were grown on Nutrient Agar (NA) (streak or pour plate) as a pure culture overnight at 30ºC. The cultures were washed with 1 ml of sterile MRD and transferred into 250ml of Nutrient Broth and incubated again at 30ºC overnight in a New Brunswick floor standing orbital shaker at 200rpm. The overnight inoculum (0.1ml samples) was spread plated on Nutrient Agar and selective Agar as per organism to be isolated (Section 3.1.3) for enumeration. The inoculum level should be between 10^2 to 10^3 cfu/ml (FSA 2009). If the counts were lower or higher than this range the whole procedure was repeated as if the inoculum is less than 10^2 cfu/ml, it may be difficult to enumerate

and observe the effect in the product. The highest level of inoculum was chosen to be 10^3 cfu/ml, because if any microbial strain is present at the time of production at a level above that, the batch would be rejected.

In order to prepare the master stock cocktail of organisms the master stock of each genus were prepared by inoculating 1 ml of freshly grown over night culture into 250 ml of Nutrient Broth and incubated again at 30ºC overnight at 200 rpm in a New Brunswick floor standing orbital shaker. The overnight inoculum (0.1ml samples) was spread plated on Nutrient Agar and selective Agar as per organism to be isolated (Section 3.1.3) for enumeration.

3.3.2 Microbial Cocktail Preparation

The isolated bacterial and Y&M as per Section 3.1.6 they were grouped together as Bacillus spp, Staphylococcus spp, yeasts and moulds. All different types of species under the same genus were grouped to understand the cumulative effect as would be found in the environment.

For a cocktail of various microorganisms 1 ml of master stock of each genus (as per 3.3.1) was inoculated into 250 ml of Nutrient Broth and incubated again at 30ºC overnight at 200 rpm in a New Brunswick floor standing orbital shaker. The overnight inoculum (0.1ml samples) was spread plated on Nutrient Agar for enumeration.

3.3.3 Inoculation Methods

For liquid products 1ml of overnight grown fresh culture was inoculated into 250 ml of the growth medium to give a stock inoculum level of a minimum of 10^2 to 10^3 cfu/ml based on finished product specifications (Table 4.1). In order to challenge test spread / semi solid products, the stab method by Holliday and Beuchat (2003) was followed. Pure isolated colonies were picked off from the Nutrient Agar plate with a sterile stick from the cocktail stock and stabbed into 10 g of the sterile sample to give a "Figure of 8" to ensure adequate organisms were transferred from stick into the sample.

The spreads were aerated in the tub using a sterile spatula for 2 min and incubated throughout their product life at room temperature 20ºC, refrigeration temperature 5ºC and at –18ºC. For oil 250g of block butter was heated at 70^0C and the oil phase decanted carefully and sterilised by autoclaving. The samples were analysed for the presence of methyl ethyl ketone by GC-MS (Section 3.2).

3.3.4 'Cheese flavour' abuse and challenge test

Spread samples were prepared as per Section 3.1.1 and plated out on NA and selective agar for various microorganisms to perform a total count prior to inoculation with the challenge microorganisms. The samples were inoculated as either a purified or cocktail of microorganisms.

For the 'cheese flavour' abuse test, the spread samples were aerated in the tub using a sterile spatula for 2 minutes and incubated throughout the product life at

room temperature 20ºC, refrigeration temperature 5ºC and at –18ºC. For the oil blend, 250g of block butter was heated at 70ºC and the oil phase decanted carefully and sterilised by autoclave. The samples were analysed for the presence of methyl ethyl ketone by GC-MS (Section 3.2). The oil blends of the spread formulation were also challenge tested with the causative spoilage agents. For oil blend, 250g of block butter were heated at 70ºC and the oil phase decanted carefully and sterilised by autoclaving. For each experiment 10ml of the sterile oil was used.

In order to perform the challenge test to be analysed via the FFA method (3.2.1.2), for liquid products (milk, cream, oil or buttermilk) 1ml of overnight grown fresh culture was inoculated into 10 ml of the growth medium and incubated at 37ºC for 7 days prior to analysing for MK as per method 3.2.1.2.

In order to challenge test spread / semi solid products, the stab method by Holliday and Beuchat (2003) was followed. Pure isolated colonies were picked off from the Nutrient Agar plate with a sterile stick and stabbed into 10 g of the sterile sample to give a "Figure of 8" to ensure adequate organisms were transferred from the stick into the sample and incubated at 37ºC for 7 (In-house accelerated abuse test) days prior to analysing for MK as per method 3.2.1.2.

3.3.5 Effect of antimicrobial agents

Three different types of medium were used in this experiment Chapex-Dox medium, OGYEA and Nutrient Agar. Different levels of the antimicrobial agent were added at different concentrations while making up a minimum of one-litre of medium (according to the manufacturer's instructions) of each type and then autoclaved at 121ºC for 15 minutes.

Table 3.3 Concentration of various antimicrobial agents

Antimicrobial Agent	Concentration
Potassium Chloride*	0.01, 0.05,0.1 & 2 (m/v) %
Sodium Chloride*	0.0, 1.0, 2.0, 5.0 &10 (m/v) %
pH*	6.5, 6.0, 5.5, 5.0, 4.5 & 4.0
Rosemary herb**	0.55 g /l

* supplied by Oxoid

** bought from a health store

Pure identified bacterial isolates were spread plated on Nutrient Agar and incubated for 24 h at 37º C. Growth was then washed off with sterile 10 ml MRD and transferred into 250 ml of sterile Nutrient Broth supplemented with an antimicrobial agent (Table 3.3) and incubated in a shaking incubator at 300 rpm overnight at 37ºC for 24 h. One ml out of the tenfold serial dilution was enumerated by pour plate in Nutrient Agar and incubated at 37º C for 24 h.

3.3.6 Listeria spp challenge test

Listeria spp isolates were isolated from products and ingredients from a dairy factory that produces cream for further processing. These were used for the challenge tests (Section 3.1). To isolate natural environmental contaminants, the factory environment such as drains, floor, walls, conveyors, fillers etc. were swabbed. Ready prepared sponge swabs (TS15-b; hydrated with 5 ml neutralising buffer supplied by Oxoid) were used. Samples were taken by wiping the swab in a zig zag motion across the surface area. The zig zags should be close together to cover as much of the surface area as possible (Figure 3.3). Once the swab was drawn over the surface area it was then re-swabbed at 90° rotation to the original swab line before being placed in the neutralising buffer (Monopotassium Phosphate 42.5 mg/l, Sodium Thiosulfate 0.16 g/l, Aryl Sulfonate Complex 5.0g/l, Oxoid) ready to be analysed (Section 3.1.3.5).

3.3.6.1 Identification of the Listeria spp

The isolates from the environment screening were identified by general microbiology in Section 3.1.3.4. For further identification the isolates were sent to the Health Protection Agency (HPA) for Ribotyping in order identify the genetic similarity between each other.

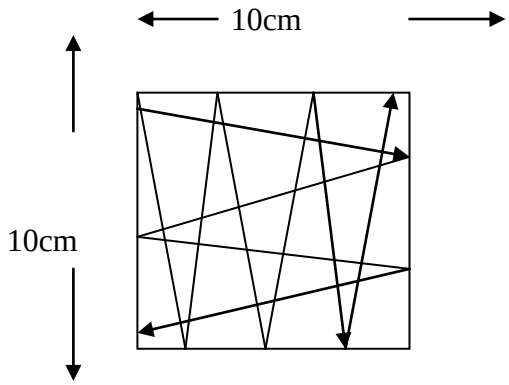


Figure 3.3 Methods of swabbing environments

3.3.6.2 Preparation of the Listeria inoculums for challenge tests

All the identified Listeria spp were grown on Nutrient Agar as a pure culture overnight at 30ºC (streak plate). The culture was washed off the plate with 1 ml of sterile MRD and transferred into 250ml of Nutrient Broth and incubated again at 30ºC overnight at 200 rpm in a New Brunswick floor standing orbital shaker. The overnight inoculum (0.1ml samples) was spread plated on Nutrient Agar and selective Oxford Agar (Section 3.1.3) for enumeration. The inoculum level should be between 10^2 to 10^3 cfu/ml (FSA 2009). If the counts were lower or higher than this range the whole procedure was repeated based on the finished product specification of the product. Also if the inoculum is less than 10^2 cfu/ml, it may be difficult to enumerate and observe the effect in the product. The highest level of inoculum was chosen to be 10^3 cfu/ml, because if any microbial strain is present at the time of production at a level above that, the batch would be rejected.

3.3.6.3 Inoculation methods

For liquid products 1ml of overnight grown fresh culture was inoculated into 250 ml of the growth medium to give a stock inoculum level of three different levels: 10, 50 and 10^2 cfu/ml.

In order to challenge test liquid products such as milk and cream 1ml of overnight grown fresh culture were inoculated into 100 ml of the growth medium and incubated at 30ºC and measured after 24 hours.

For a spread / semi solid product, the stab method by Holliday and Beuchat (2003) was followed. Pure isolated colonies were picked off from the Nutrient Agar plate with a sterile stick and stabbed into 10 g of the sterile sample to give a "Figure of 8" motion to ensure adequate organisms were transferred from stick into the sample.

3.3.6.4 Listeria monocytogenes emulsion challenge test

Listeria spp isolated from the dairy environment were challenge tested at variable of salt and pH levels. To adjust the salt concentration of the emulsion NaCl (Oxoid) was used at the level of 0.0, 0.5, 1.0 and 1.5 % in a 500g emulsion cream, the pH was altered by adding the required volume of HCl or NaOH (Oxoid). The details of the composition are in Table 3.4. The isolated strains were inoculated as per 3.3.6.3 and incubated at 30º C and the growth was measured as a count (cfu / g) over a 75 days (1800 hours) period as per the spread shelf life.

Table 3.4 Variable emulsion medium for challenge Test

Constant Parameter	Variable Salt	Variable pH	Variable a_w
Salt 1.5%		5.5, 6.0, 6.5	
pH 5.5	0.0, 0.5, 1.0, 1.5		0.93, 0.95, 0.98
a_w 0.95		5.5, 6.0, 6.5	

3.3.6.5 Listeria monocytogenes spread challenge test

Challenge testing on a finished packed spread sample was carried out at Campden BRI spread and butter specialist microbiology department. Overnight suspensions of each of the organisms were prepared in 500ml volumes of nutrient broth containing glass-bumping granules, the organisms adhere to the surface of the beading to come in contact with the matrix directly once used. The suspensions were prepared at 37°C and after incubation the broth was drained away and the beads decanted into large petri dishes with filter paper for removal of excess moisture. Each individual bead was then used as an inoculum point within the product.

At the predetermined sampling point each bead within a 1g mass of spread was removed from the sample and placed into a 9ml volume of warmed tri-sodium citrate diluent. This constituted the primary dilution of the inoculum. Serial dilutions were then performed and a range of dilutions plated out in order to determine the level of relevant inoculum. Standard microbiological techniques

were adopted for enumeration using Oxford agar to enumerate the Listeria inoculum

3.3.6.6 Listeria spp heat resistance test

Wild strains of Listeria spp isolated from the factory environment along with L. monocytogenes NCTC 11994 used as a control. A known level of isolate was inoculated into 50ml of whole milk and then refrigerated at 4ºC for 48 hrs to allow adaptation.

Samples of 50 ml or g were inoculated with levels of 6.0 x 10^4 cfu/ml of culture. Each of the inoculated samples was exposed to the following heat treatment using a water bath that had already reached the target temperature. Samples were timed after they reached the desired temperature and were quickly cooled down afterwards using an ice bath. It took between 20 to 30 minutes for the temperatures to achieve the appropriate temperature and then held for the respective time.

- 65ºC held for 25 seconds
- 72 ºC held for 25 seconds
- 73.6 ºC held until the temperature was reached
- 78 ºC held for 25 seconds

Both enumeration and detection were used to determine if very low levels of cells or sub-lethally injured cells were present post heat treatment that may not be recoverable using standard culture enumeration/plating (Section 3.1).

3.3.6.7 Acid and alkali resistance of Listeria spp

This study had the aim of assessing if wild strains of Listeria monocytogenes (isolated from both raw milk and final product samples) were resistant to the following environmental conditions;

- 0.8% alkali solution held at 80ºC for 40 minutes
- 0.6% acid solution held at 50ºC for 12 h
- 0.6% acid solution held at 50 ºC for 24 h

The inoculum from 3.3.3.1 from the Oxford Agar (Oxoid) was refrigerated for 7 days to create the dehydrated (dry-stressed) culture. After 5 days the same inoculmn of L monocytogenes was grown up in 250 ml of Nutrient Broth overnight at 30ºC. After overnight incubation the inoculant was plated out onto Oxford Agar and incubated overnight at 30°C to create a fresh plate culture. Both fresh and dehydrated cultures were used to determine the difference in the acid or alkali resistance of fresh or dry-stressed cells. Using a 10µl culture loop the culture was swept up and used to inoculate 500 ml of sterile Nutrient Broth at a target level of $10^7 – 10^8$ cfu / ml (estimated by enumeration method) and the pH of

the broths were altered by adding the required volume of HCl or NaOH whilst constantly mixing the broth to create the 0.8% and 0.6% acid / alkali solutions.

The inoculated broths from both the fresh cultures and dehydrated cultures were treated for the following times and temperatures, 0.8% NaOH solution –was incubated at 80ºC for 40 minutes, 0.6% HCl solution –was incubated at 50ºC for 12 h and 0.6% HCL solution was incubated at 50ºC for 24h. The samples were rapidly cooled to room temperature before being analysed for Listeria spp for both enumeration and detection method as per Section 3.1.3.

3.3.6.8 Salt tolerance of Listeria spp

Listeria monocytogenes isolated from dairy processing environments and products (3.1.6) were challenge tested for various salt tolerances at different levels. Sodium Chloride (NaCl), Potassium Chloride (KCl) and Calcium Chloride ($CaCl_2$) were used at the levels of 0.0, 0.5, 1.0, 1.5 and 2.0%. The salts were all sourced from Oxoid. Milk Plate Count Broth was used for this experiment as per the formulation given in Table 3.5 at pH 6.9 ±0.1.

Table 3.5 Milk Plate Count Broth Composition (Oxoid)

Ingredients	Weight (g/l)
Tryptone	5.0
Yeast Extract	2.5
Glucose	1.0
Skimmed Milk Powder	1.0

An inoculum of 1 cfu/ml Listeria monocytogenes (prepared as in Section 3.3.3.1) was inoculated in 250ml of the Milk Plate Count Broth and incubated in a shaking incubator at 300 rpm in a Sartorius shaker at 30°C and optical density was measured of 1ml sample every 30 minutes for 10 to 12 hrs using a Pharmaci Biotech Ultrospec-2000 spectrophotometer using a wavelength of 600 nm for bacterial cells. Growth curves were also produced from a microtitre plate format using a Tecan GENios Pro that is a multifunctional microplate reader that can read a plate of 96 wells over a period of time. Each well was inoculated with 200µl of final volume of the mixture of one of the test broths and the bacteria (with a positive OD value) and incubated at 30ºC for 24 h. Each individual well was read every 30 minutes with a 5 seconds shake at 100 rpm beforehand. The optical density was measured at 600 nm. The same tolerance experiment was carried out using the liquid phase of the spread, compositional analysis as per Table 3.6 to evaluate the growth level in the product.

Table 3.6 Bulk liquid phase composition

Ingredients	Total Weight (kg)
Water	45.307
Salt	1.100
Buttermilk Powder	1.000
Starch	2.000
Potassium Sorbate	0.125
Lactic Acid	0.055
Vitamin B	0.031

CHAPTER 4 MICROBIOLOGY RESULTS

Each batch of finished spread production is tested daily to ensure the product complies with company and EU microbiological regulations (2073/2005) for the level of microbes present. The products are released to the market if the TVC, Enterobacteriaceae and Yeast and Mould and specific pathogen test results are within the product specification guideline (Table 4.1) in order to verify correct processing (pasteurisation) for product safety and quality.

4.1 INTRODUCTION

To establish the root cause of spoilage in spreads, retained samples showing 'cheese flavour' development were analysed together with fresh ingredients and finished products to isolate and identify the microbial contaminants. The main ingredients that are used in spreads' production are skimmed milk powder, sweet cream, sweet cream buttermilk, cream, buttermilk, oil, water, salt and emulsifiers. To identify the potential source of the contaminants, the factory environment such as the air and water were also tested.

Table 4.1 Typical Finished product specification

Typical Finished Product Specification		
Parameter	Target (cfu/g)	Reject (cfu/g)
TVC	<1000	>10,000
Enterobacteriaceae	<10	>100
Yeasts	<10	>20
Mould	<10	>20
Salmonella spp	Absent	Present
Listeria spp	Absent	Present
Bacillus spp	<100	>1000
Staphylococcus spp	<20	>50
E.coli	Absent	Present

4.2 MICROBIOLOGY ANALYSIS

4.2.1 Identification of microbial contamination

The identification of the causative spoilage microbes in the spread was carried out using both culture and molecular techniques. A variety of microbial strains were examined in the products, ingredients and the factory environment. The array of tests carried out were TVC, Enterobacteriaceae, Listeria spp, Salmonella spp and E.coli in order to identify the root cause of the spoilage of the 'cheese flavour' or taint microbes. A total aerobic count (TVC Section 3.1.2) was performed for an indication of the total microbial or contaminant level in a spoiled product vs.

standard product. Selective agars such as VRBGA for Enterobacteriaceae (Section 3.1.3.1); TBX for E.coli (Section 3.1.3.2); XLD for Salmonella spp (Section 3.1.3.3); PEMBA for B. cereus (Section 3.1.3.4); BPA for Staphylococcus spp (Section 3.1.3.5); Oxford for Listeria spp (Section 3.1.3.6); C-F-C for Pseudomonas spp (Section 3.1.3.7); caseinate medium for the proteolytic organisms (Section 3.1.3.8) and TBA for lipolytic organisms (Section 3.1.3.9); MRSA for lactic acid bacteria (Section 3.1.3.10) and Chapex-Dox, OGYEA for moulds and yeasts (Section 3.1.3.11) respectively were used for initial screening of the microbial loads in the products.

A total of 100 spread samples packed in 500 g tubs were analysed from April 2009 to October 2009, of which 85 had 'cheese flavour' development (Table 10.1 sample numbers 1 to 85) and 15 were standard freshly produced products (Table 10.1 sample number 86 to 100). Among the ingredients skimmed milk, sweet cream, sweet cream buttermilk, cream, buttermilk, oil and water were sampled. Each sample was screened weekly over a three month period. For traceability reasons sampling was performed on product with relevant ingredient batches and intermediate processed samples stored at silos, filler samples and finished products.

Appendix 10.4 Table 10.1 summaries the result of the total number of microorganisms enumerated from 100 products. The samples were prepared as per Section 3.1.1 and were microbiologically analysed by plating directly onto selective and non-selective media by the methods in Sections 3.1.2 and 3.1.3. At

this stage of analysis both presence/absence and enumeration were performed to give both qualitative and quantitative results.

The data from Table 10.1 (Appendix 10.4) were further summarised in Table 4.2, clearly indicating that the 'cheese flavour' samples had a higher percentage of Bacillus spp, Staphylococcus spp, yeast and mould strains and lipolytic bacteria. The TVC counts in the standard spreads were $\leq 10^2$ cfu/g compared to the 'cheese flavour' samples, which ranged from 10^2 - 10^5 cfu/g. This indicates post process contamination in the product. Enterobacteriaceae, Pseudomonas spp and lactic acid bacteria (LAB) were found at varying levels between 10 cfu/g to 10^3 cfu/g in products but were not of concern with respect to food safety as per EC 2075/ 2005 and Public Health England (2009) Guidelines for Assessing the Microbiological Safety of Ready to Eat Foods Placed on the Market. The 'cheese flavour' products had a higher number of Enterobacteriaceae, Pseudomonas spp and LAB compared to those of standard ones at a range from 10^2 cfu/g to 10^4 cfu/g which were within one log of variance from the standard finished spread product (one log is the standard uncertainty of measurement of the microbiology laboratory).

Bacillus spp in the 'cheese flavour' samples varied from 10^2 to 10^6 cfu/g. Table 4.2 shows 13% of the standard samples were positive for Bacillus spp. Table 10.1 (Appendix 10.4) showed that the total number of positive samples with Bacillus spp in standard spread samples were very low (only 2 positive out of 15) but the levels were within the product specification (Table 4.1). On the other hand the

levels in the ‘cheese flavour’ samples were mostly greater than 10^2cfu/g (i.e. above product specification guideline); some of them were as high as 10^5 cfu/g, which was of concern, as per Public Health England the guidance in RTE food, a level exceeding 10^6 can be of concern for food poisoning outbreaks (Public Health England 2009).

Only 1 out of 15 standard samples was positive for Staphylococcus spp with a count of 110 cfu/g compared to ≤50 cfu/g in the product specification. But the ‘cheese flavour’’ samples contained Staphylococcus spp at 10^2 to 10^3 cfu/g levels. Although the levels are much lower than the levels of concern for staphylococcal toxin production (10^4 cfu/g), they were still higher than normal. Usually the presence of higher levels of Staphylococcus spp indicates a process hygiene issue (≥ 20 cfu/g EU Micro Criteria 2005).

The total numbers of yeasts and moulds in the samples were not as high as the bacterial species. The maximum number of yeasts was only 250 cfu/g and moulds were 200 cfu/g. But some of the moulds could have spores present in the sample, which were not growing in the product but may increase the counts. Only 71 of the ‘cheese flavour’ samples were positive for yeasts and moulds out of 85. All the 71 samples showed a strong smell of ‘cheese flavour’’ compared to the other 14 ‘cheese flavour’ and 15 standard samples.

All 100 samples were checked for the day, date and time of production. One batch of spread production is a 72-hour run. The factory is deep cleaned (CIP) every 72

hours. It was noted that all the standard samples with low level of contaminants were produced within the first 24 hours of the 72-hour run. On the other hand the 'cheese flavour' samples varied over different days of production within the three-day batch period. But 78% of the positive samples were produced within the first six to twelve hours of the three-day run, indicating an increased level of cross contamination just after the deep cleaning of the factory and the machines that indicates aerosol cross contamination of the finished product within the filler area. Moreover the site pasteuriser runs for 10 hours after which it requires a full CIP taking approximately 4 hours. Hence in a 72 hour run the site will process 24 hour run of product that may be carried over depending on the run rate and the break downs. Hence the age of the raw ingredients and processed emulsions increases.

Table 4.2 Summary of microbiology results of 100 spread samples.

Microbiology Analysis	Positive Result Total		% Positive	
	'cheese flavour' Samples	Standard Samples	'cheese flavour' Samples	Standard Samples
E.coli	0	0	0	0
Salmonella spp	0	0	0	0
Micrococcus spp	0	0	0	0
Listeria spp	0	0	0	0
Enterobacteriaceae	20	1	24	7
Pseudomonas spp	18	5	21	33
Proteolytic Bacteria	7	0	8	0
Lactic Acid Bacteria	7	0	8	0
Bacillus spp	63	2	74	13
Staphylococcus spp	62	1	73	7
Lipolytic Bacteria	80	2	94	13
Yeasts & Moulds	71	0	84	0

4.2.2 Isolation of microbes from products

4.2.2.1 Phenotypic characterisation of the strains from spreads

Following the presumptive identification in a presence/absence test in Section 4.1.1 (Table 4.2) the bacterial strains isolated from the 100 finished packed spread samples (of which 85 were 'cheese flavour' samples and 15 were standard) were further characterised by colony morphology, Gram staining, oxidase and catalase tests to determine presumptive identification of the microorganisms (Section 3.1.2) (Table 4.3). The Bergy's Manual 2nd Edition (2010) was used as a reference to group the microorganisms based on their biochemical test results.

Table 4.3 Characteristics of microorganisms isolated from samples

Group	Cell Morphology	Gram Stain	Catalase	Oxidase	Presumptive Identification	% Positive in Products
1	Rods	Positive	Positive	Variable	*Bacillus spp*	74
2	Cocci	Positive	Positive	Negative	*Staphylococcus spp*	73
3	Cocci	N/A	N/A	N/A	Yeast	84
4	Irregular/ rhizoidal	N/A	N/A	N/A	Mould	84
5	Rods	Negative	Positive	Negative	Enterobacteriaceae	24
6	Rods	Negative	Positive	Positive	*Pseudomonas spp*	21
7	Rods	Positive	Negative	Negative	Lactic Acid Bacteria	8

Group 1 contained a Gram-positive spore forming strain that appeared rod shaped under oil immersion microscopy. The bacteria were presumptively identified as Bacillus spp due to the presence of endospores. 74% of the 'cheese flavour' samples had Bacillus spp contamination compared to only 13% of the fresh packed spread products. They were further confirmed by API 50CHB (§4.2.2.2) and 16S rDNA (§4.3). Group 2 was presumptively identified as Staphylococcus spp being Gram-positive, catalase positive appearing as a cluster of cocci under

the microscope. The strains appeared as pink-yellow colonies with yellow zones on MSA by fermenting mannitol. 73% of the ‘cheese flavour’ samples had Staphylococcus spp contamination compared to only 7% of the fresh packed spread products. They were further confirmed by API Staph (§4.2.2.2) and 16S rDNA (§4.3) tests. Groups 3 and 4 contained yeast and mould. The former showed distinctive large creamy gluey colonies in OGYEA and showed budding under the microscope (wet mount). On the other hand the moulds showed a rhizoidal growth with spores on the Czapek Dox agar. Some of the colonies were white and green coloured. When observed under the microscope by performing a wet mount, the obvious presence of mycelium was noted. Yeast and mould (Y&M) colonies were further identified using 26S PCR analysis (§4.3); 84% of the ‘cheese flavour’ samples had Y&M contamination compared to none on the freshly packed spread products.

These identified strains from the products were used as a set of reference cultures and were later used to group and in identifying the microorganisms present in the ingredients and environment.

E.coli, Salmonella spp, Micrococcus spp, Listeria spp and proteolytic bacteria were absent in the standard samples with a small percentage containing occasionally very low levels of Enterobacteriaceae, Pseudomonas spp and lactic acid bacteria. Isolates from the Enterobacteriaceae, Pseudomonas spp and lactic acid bacteria plates were grouped into categories 5, 6 and 7. They are part of the general micro flora of the product.

Bacillus spp, Staphylococcus spp, lipolytic bacteria and yeasts and moulds were present at a much higher frequency compared to those of standard products and the finished product specifications (Table 4.1 and 4.2). These were referred to as the 'presumptive causative organisms' showing high levels of lipolytic activity.

4.2.2.2. API Identification

For further confirmation of Bacillus spp, API 50 CHB (bioMerieux, Inc.) was used for identifying Gram positive catalase producing sporing rod-shaped isolates (§3.1.5.1) and API STAPH (bioMerieux, Inc.) for the genera Staphylococcus and Micrococcus spp (§3.1.5.2). Table 4.4 summarises the results of identifying Bacillus spp and Staphylococcus spp and their profiles. For further identification, a molecular technique, sequencing the V3 region of 16S rDNA, (§ 4.2) was used.

On the API 50 CHB strip, the Bacillus spp isolates produced ERYthritol, D-RIBose, D-XYLose, D-FRUctose, D-MaNnosE, L-SorBosE, D-MELibiose, D-SACcharose, D-TREhalose, INUlin, D-TAGatose, Potassium GlucoNaTe, Potassium 2-KetoGluconate, Potassium 5-KetoGluconate. Staphylococcus isolates in API STAPH produced Fructose, Glucose, Maltose, Lactose, Trehalose, Mannose, Sucrose.

The API t-index was relatively low for most strains suggesting identification was not typical of the species obtained. Because of this the API speciation was confirmed with 16S rDNA sequence analysis in Section 4.3.1.

Table 4.4 API identification of Bacillus spp and Staphylococcus spp.

API KIT	Significant taxa	% ID	T	Profile
API 50 CHB	*B. subtilis*	96.8	0.67	- + - - + + - - - - - + + + - - - + + + - + + + + + + + + + + - + + + + - + + + - + + - - - - - - - - - + - - - + - - - - + + +
	B. licheniformis	99.9	0.93	- + - - + + + - - - + + + + - + - + + + - + + + + + + + + + + - + + + - - + + + - - + + - + - + - - - - + - - + + - - + - - - + + +
	B. cereus	95	0.96	- + - - - + - - - - - + + + - - - - - - - - + + + + + + + + - - + + - - - + + - - - - - - - - - + - - - + - - + - - - - + + +
API STAPH	*S. aureus*	97	0.74	6 7 3 6 1 5 3
	S. warneri	77.5	0.85	6 2 1 0 1 1 2
	S. sciuri	77	0.94	6 7 3 6 1 5 0
	S. intermedius	98.2	0.72	6 7 3 6 1 5 3

4.3 MOLECULAR IDENTIFICATION OF THE MICROBES

The presumptive colonies identified in Section 4.2 were grouped based on their colony morphology, biochemical and API test results. Further characterisation by DNA sequencing was performed on 25 bacteria and 20 yeast and mould strains.

4.3.1 16S rDNA PCR

To perform 16S rDNA sequencing, samples were prepared and analysed as per Section 3.1.6. Figure 4.5 shows the PCR product obtained with primers V3R and V3F that amplified the variable V3 region of the 16S rDNA of the bacterial strains to form products with 200 kb. Samples were separated on an agarose gel; a 100 bp marker was used. The intensity of the visible bands corresponding to the PCR product varied among samples.

The bands containing the PCR products were aseptically excised from the agarose gel, purified (method as in Section 3.1.6.1) and sent for sequencing. BLAST matches of the sequences obtained from amplicons were performed to retrieve the most closely related species from the NCBI chromosome gene bank. For bacterial isolate identification, 50 isolates from both product and environment were analysed. All 25 samples gave a satisfactory result of a match of greater than 97% base pair matching in all cases as per Tables 4.5 and 4.6.

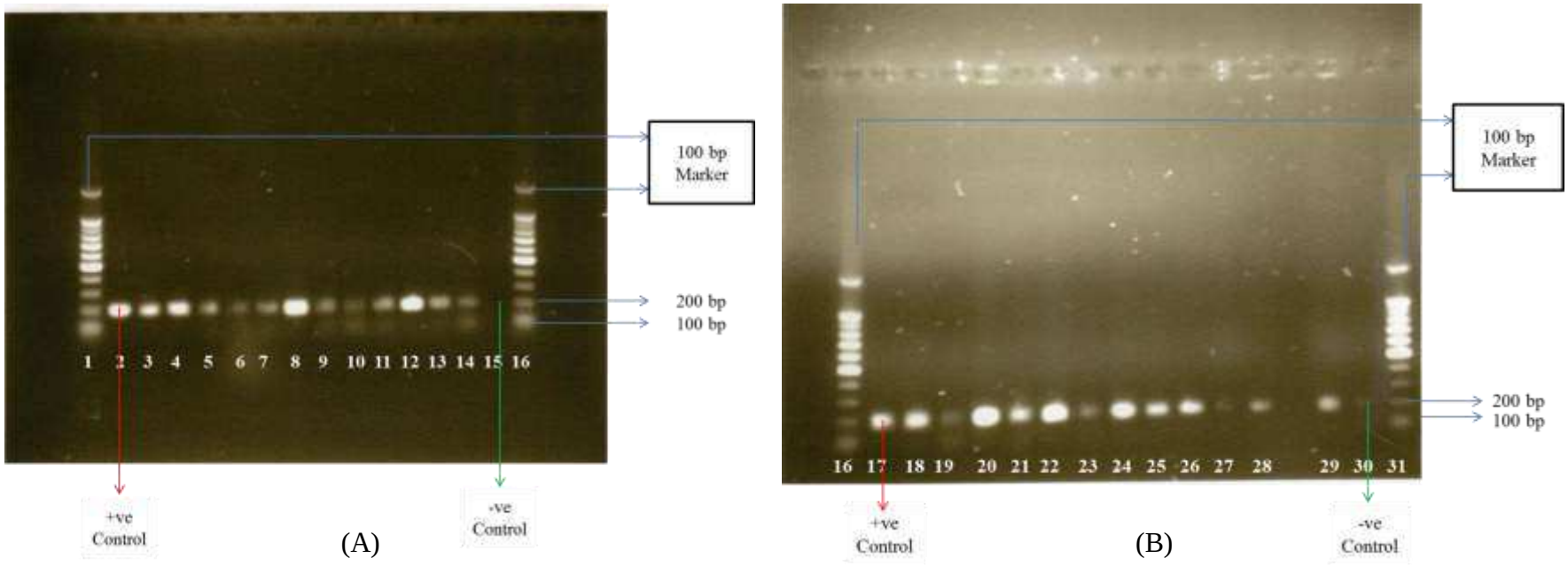


Figure 4.1 PCR products (200 bp) of amplified variable V3 region for presumptive Staphylococcus spp and Bacillus spp.

(A) Lane 2 and 15 contains positive and negative controls (no DNA) respectively and 100 bp marker in Lane 1 and 16. Lanes 3 to 10 contain Staphylococcus spp (S) and Bacillus spp (B) in lane 11 to 14. Figure 4.1 B shows Lane 17 and 30 contain positive and negative control (no DNA) respectively and 100 bp marker in Lane 16 and 31. Lanes 20 to 29 contain Staphylococcus spp (S) and Bacillus spp (B) in lane 17 to 19 the samples were run on 1% (w/v) agarose gel in 1X TAE buffer at 70V for 2 hours.

The identified species were Staphylococcus equorum, Staphylococcus pasteuri, Staphylococcus sciuri, Bacillus subtilis and Bacillus licheniformis with the detailed % bp matching stated in Tables 4.5 and 4.6 (Figure 4.1).

Table 4.5 Microorganisms identified in 16S rDNA PCR in Figure 4.1 (A)

Gel Lane	Species Identified	% bp Matching
1,16	Markers (100 bp)	
2,15	+VE & -VE CONTROL	
3	*S. sciuri*	98
4	*S. scuiri*	100
5	*S. scuiri*	94
6	*S. equorum*	96
7	*S. pasteuri*	99
8	*S. pasteuri*	99
9	*S. equorum*	99
10	*S. pasteuri*	99
11	*B. subtilis*	100
12	*B. subtilis*	100
13	*B. pumilis*	98
14	*B. licheniformis*	100

Table 4.6 Microorganisms identified in 16S rDNA PCR in Figure 4.1 (B)

Gel Lane	Species Identified	% bp Matching
16,31	Markers (100 bp)	
17,30	+VE & -VE CONTROL	
17	*B. subtilis*	100
18	*B. subtilis*	100
19	*B. cereus*	98
20	*S. equorum*	96
21	*S. pasteuri*	99
22	*S. equorum*	98
23	*S. equorum*	100
24	*S. equorum*	90
25	*S. equorum*	92
26	*S. equorum*	95
27	*S. equorum*	98
28	*S. equorum*	99
29	*S. equorum*	100

4.3.2 26S rDNA PCR

Figure 4.2 shows the PCR product obtained with primers NL1 and reverse LS2 that amplified the variable region of the 26S rDNA of the Y&M strains. The intensity of the visible bands corresponding to the PCR product varied among samples.

The bands containing the PCR products were aseptically excised from the agarose gel, purified and sent for sequencing. BLAST matches of the sequences obtained from amplicons were performed to retrieve the most closely related species from the NCBI chromosome gene bank. Pichia guilliermondii, Candida guilliermondii, Candida parapsilosis and Candida ortho parapsilosis were identified with more than 98% base pair matching.

Pichia guilliermondii is a species of yeast of the genus Pichia whose asexual or anamorphic form is known as Candida guilliermondii. Candida guilliermondii has been isolated from numerous human infections, isolated from normal skin, faeces of animalsetc. Pichia guilliermondii is widely distributed in nature (routinely isolated from insects, soil, plants, atmosphere, seawater, the exudates of various trees, and processed foods) and is a common constituent of the normal human micro flora. It can also come from packaging. Lipolytic activity was observed in 24 out of 29 of the isolated strains and was not a determinant for attaining high levels of contamination.

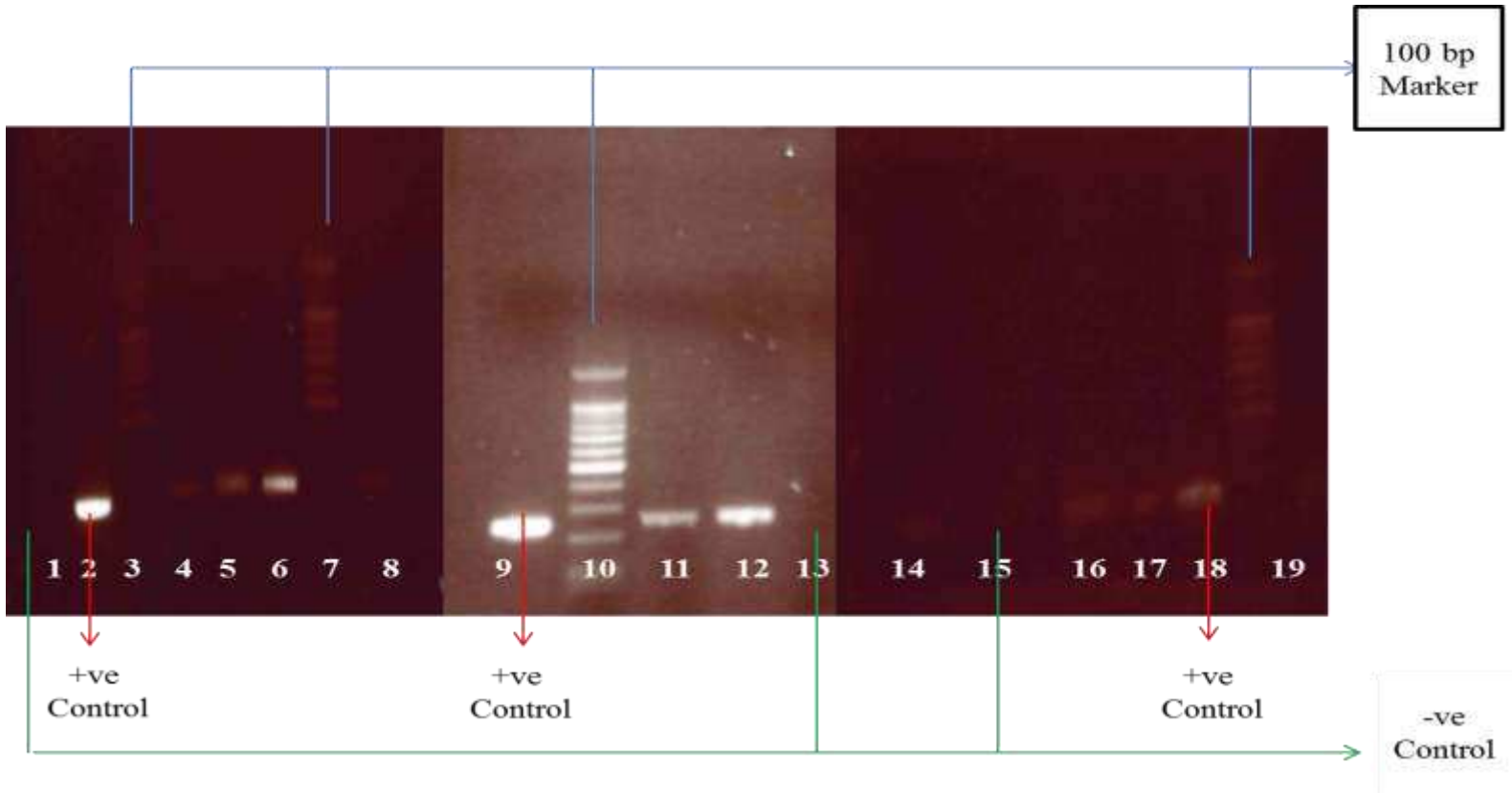


Figure 4.2 PCR products (200 bp) of the amplified variable region for presumptive yeast strains and 300 bp for moulds.

Lanes 1, 13 and 14 contain negative control (no DNA) and 2, 9 and 18 positive control. Lanes 3,6,8,16 contains yeast (Y) strains and moulds (M) are in lanes 4,5,7,11,12,13,14 and 17. The samples were run on 1% (w/v) agarose gel in 1X TAE buffer at 70V for 2 hours

The moulds identified were Penicillium brevicompactum and Penicillium crocicola with more than 96% base pair similarity (Table 4.7). P. brevicompactum is xerophilic in nature and can spoil refrigerated products such as cheese, margarine and dairy products (Pitt and Hocking 2009).

Table 4.7 Microorganisms identified in 26S rDNA PCR in Figure 4.2

Gel Lane	Species Identified	% bp Matching
3,7,10	Markers (100 bp)	
2, 9 & 18	+VE CONTROL	
1, 13 & 14	-VE CONTROL	
3	*C. orthopsilosis*	99
4	*P. quilliermondii*	98
5	*P. crocicola*	99
6	*C. orthopsilosis*	96
7	*P. quilliermondii*	100
8	*C. orthopsilosis*	94
11	*P. brevicompactum*	98
12	*P. crocicola*	96
13	*P. brevicompactum*	100
14	*P. quilliermondii*	100
16	*C. orthopsilosis*	99
17	*P. quilliermondii*	100

4.4 FURTHER ANALYSIS

To identify the main source of contaminants the product ingredients such as skimmed milk, sweet cream, sweet cream buttermilk, cream, buttermilk, oil and potable water along with the environmental processing air and the packaging were further analysed for the isolates identified in Table 4.2 and 4.3.

4.4.1 Ingredients analysis

The ingredients of the spread formulation were further analysed and prepared as per Section 3.1.1. They were screened for the presence of Bacillus spp, Staphylococcus spp, yeasts and moulds as per Sections 3.1.2 and 3.1.3 based on the findings in Section 4.2 and 4.3.

The ingredients were screened daily over three months to cover different batches of ingredient supply. In order to run a traceability exercise to relate the source of the microbial contaminant in the finished product with the ingredients, sampling was performed from the intermediate processed sample to the filler and finally finished product.

The high-risk ingredients in the product were the buttermilk, skimmed milk, sweet cream buttermilk and cream being highly perishable products because of the nature of the product composition (presence of fat, water and sugar). A high percentage of samples of buttermilk, sweet cream buttermilk and skimmed milk

contained Bacillus spp (66%), Staphylococcus spp (60%), yeasts (80%) and a few contained moulds (Table 4.8).

The overall contamination level in the processed emulsion contained 69% Bacillus spp, 56% Staphylococcus spp, 79% yeasts and 35% mould compared to that of the finished product of the filler. As the silo holds 120,000 l of product over the 72-hour run-period before being cleaned (CIP), it is likely to get higher frequency of microbial contamination compared to the small volume of fresh samples from the filler as they are heat treated prior to filling.

The presence of the causative organisms Bacillus spp, Staphylococcus sppand yeasts was reduced by 50% in the finished product (Table 4.8) as compared to the levels in the ingredients other than mould. This was obvious as pasteurisation give a minimum of 5- 6 log reduction of the total microbial count (Andrew et al. 2003).

As for the products, the age of the ingredients was also noted while analysing for the presence of microbes. It was observed that if any of the high-risk ingredients were more than 42-hours old from the last pasteurisation, the microbial load significantly increased by at least one log count that as per EU micro criteria indicating that the product was able to support the growth of the organism (CCFRA guideline 52).

Table 4.8 Microbiology analysis of the ingredients in a three month period.

Sample	Sample Number	% Positive samples with microbial group				
		Bacillus spp	*Staphylococcus* spp	Yeasts	Moulds	Lipolytic microorganisms
Buttermilk	176	66	65	73	17	19
Skimmed Milk	124	67	59	100	8	80
Cream	143	59	38	45	15	14
Sweetcream Buttermilk (pre-pasterurisation)	50	71	68	68	45	80
Sweetcream Buttermilk (post-pasterurisation)	50	55	20	15	35	40
Oil	30	10	5	0	0	0
Water	35	0	0	0	0	0
Silo	33	69	56	79	35	10
Finished product	31	12	6	16	15	5

The lipolytic activity (§3.1.3.7) of isolates from the ingredients and relevant finished products were analysed, as it was observed (Appendix 1) that all of the isolated strains from the 85 'cheese flavour' product samples (Bacillus spp, Staphylococcus spp, yeasts and moulds) showed lipolytic activity (Appendix 10.4, Table 10.1). Lipolytic microorganisms are capable of producing lipase enzymes under favourable conditions. The lipolytics (BS 4287:1989) show a distinctive clearing zone around the colonies in the TBA medium. The lipase enzyme hydrolyses glycerides to form free fatty acids; in butter and other dairy products this can cause rancidity. The characteristic odours of rancid dairy products are caused by the release of butyric acid - a volatile fatty acid.

From Table 4.8 it was noted that 80% of the skimmed milk and sweet cream buttermilk (pre-pasteurisation) samples were positive for lipolytic microorganisms. The percentage of lipolytics nearly halved in the sweet cream buttermilk after pasteurisation, which may indicate that the main contaminant is Bacillus spp, which are spore formers and can survive the pasteurisation

temperature. The cream and buttermilk had lower numbers of lipolytics 14 and 19% respectively, but the total percentage of contaminated samples with the presence of Bacillus spp was a lot higher with a % positive of 59 and 67 respectively.

The main lipolytic microorganisms found in the process ingredients were mainly Bacillus spp, Staphylococcus spp and some moulds as identified in Section 4.2 and 4.3. Most of the ingredients, more than 50 percent, were positive with the presumptive causative spoilage organisms. The oil and water were of lowest risk, as it would be assumed, due to the nature of these products.

The ingredients that showed the presence of the causative species are all a bi-product of the site from processing milk and cream. Therefore the environment of this factory was screened for the presence of the same microbes in order to find the route of contamination.

4.4.2 Environmental air sampling

The spreads factory operates in a unilateral flow for GHP and GMP. The intake bay and raw storage silo are situated outside the main processing area giving a physical segregation to prevent cross contamination betweenraw and processed product (Figure 4.3). The raw cream and other ingredients for spread production are transferred to the processing hall pasteuriser or mixing tank via a valve matrix through pipe work. After pasteurisation the products are then transferred into an intermediate storage silo and then gravity fed to the packing lines for finished

product packing. Packaging are fed directly to the line from the packaging store and the end product is transported to a cold store via automated lines and packed into pallets ready for despatch. The general process schematic is detailed in Figure 4.3.

The factory air was sampled for three months, six times a day to cover every shift, process and production change and the effect of cleaning of the environment. The environment was also analysed to identify the root cause of spoilage. Mainly settle plates were used throughout the factory (Figure 4.3) as per method 3.1.4.4 in the Intake Bay (storage silo, holding tanks, goods in-house); Process Hall (balance tank, homogeniser, pasteuriser, churner, salt solution); Benhill or Packing Area (packaging storage area, filler or leider hall, finished pack area) and Storage Area i.e. dry goods store, cold store and despatch. For Bacillus spp, Staphylococcus spp and moulds and yeasts plates were left in the environment for 2 hours to capture the aerosols. Sometimes an air sampler was also used to draw a minimum of 1000l of air onto the open plates (Section 3.4.1.1).

After comparing 500 plates over two weeks it was noticed that the settle plates picked up less air contaminants compared to the air sampler method. The colony types observed on the settle plates compared to the air drawn plates were fewer in numbers. Also a few settle plates were misplaced and spoiled while they were left in the factory. The most inconvenient sampling point was the filler area. When the plates were left for two hours, they were either contaminated with product, or got soaked by filler sanitizer / hose pipe or got overturned. Therefore from two weeks

onwards, air was sampled only using an air sampler. The volume 1000l is a standard used by the industry. A variation of the volume (1500, 2000 and 2500l) was also tested to find the optimum volume for efficient screening, but 1000l seemed the better option. The predominant flora on the environment plates was a variety of yeasts and moulds that looked morphologically similar to those of the product and ingredients. All the other microorganisms such as Enterobacteriaceae, E.coli, Salmonella spp, Bacillus spp, Staphylococcus spp, Micrococcus spp, Listeria spp, Pseudomonas spp, proteolytic and lactic acid bacteria were absent. All the strains of yeast and mould also showed similar levels of lipolytic activity as those of the product and ingredients (§ 4.2.2 and 4.4.1). Figures 4.4 and 4.5 shows the weekly trends in yeast and mould and combined Y&M counts in different sectors of the processing plant. The 'x' axis denotes the total number of tests performed over the period covering the 72-hour production day and 'y' axis denotes colony count per litre of air drawn (cfu/l).

In Figures 4.4 and 4.5 high peaks of counts were observed on day 1 of most production dates over 3 months. This indicates the contamination to be most likely in the environment; hence the levels peak during the deep clean of the factory. The factory runs continuously for 72 hours, after which a deep clean is performed and the next batch starts on the following morning at 6 am On the first day of production of each batch, high levels of yeasts and moulds were observed in the environment from the air plates and the levels slowly dropped off in the three-day period.

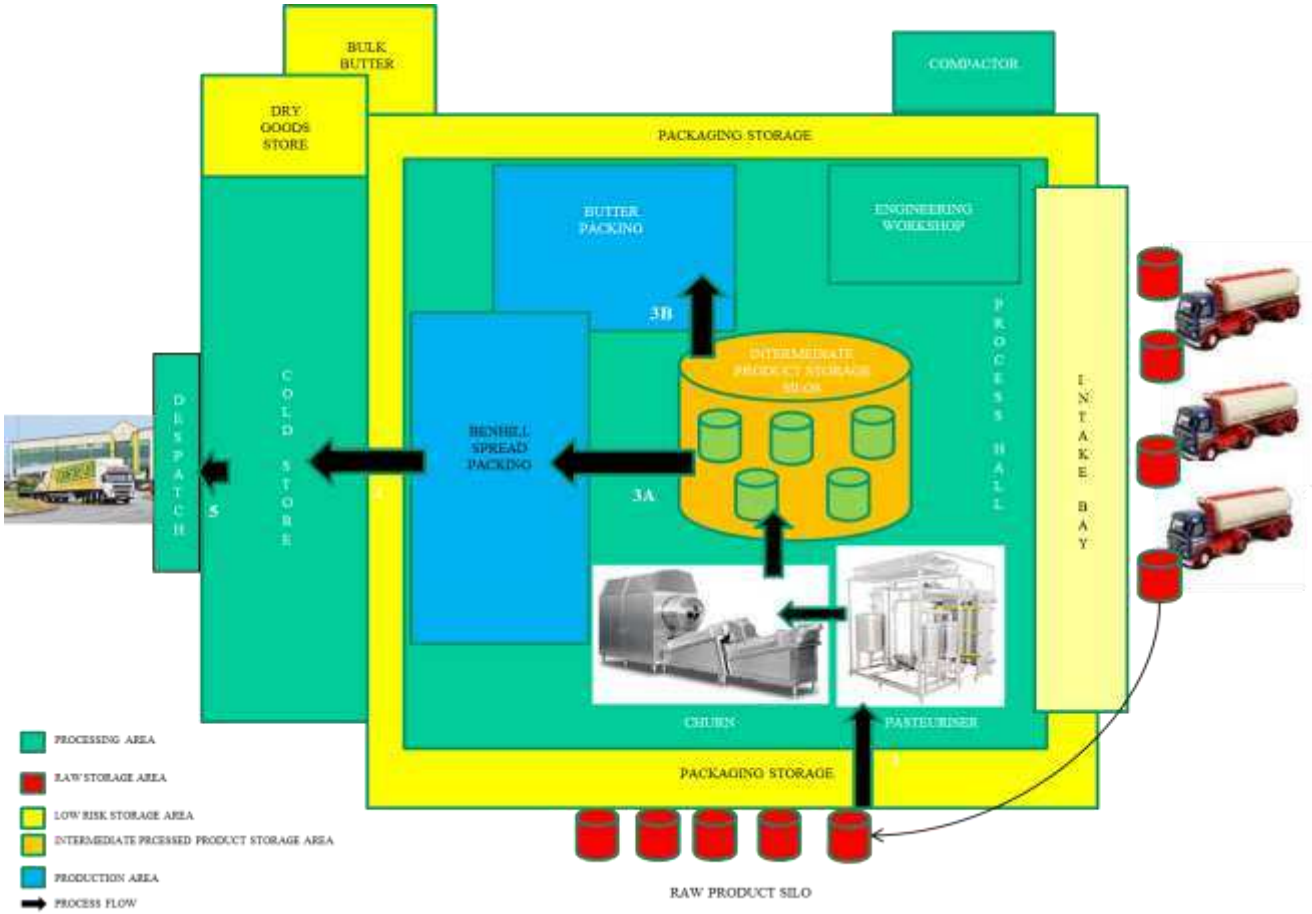


Figure 4.3 Spread Processing Plant Layout outlining the Intake Bay, Process Hall, Benhill or Packing and Storage Area i.e. dry goods store, cold store and despatch. The flow starts at step 1 in the intake bay, followed to flow 2 in the process room and then the filling hall (3) and finally to coldstore (4) and despatch (5)

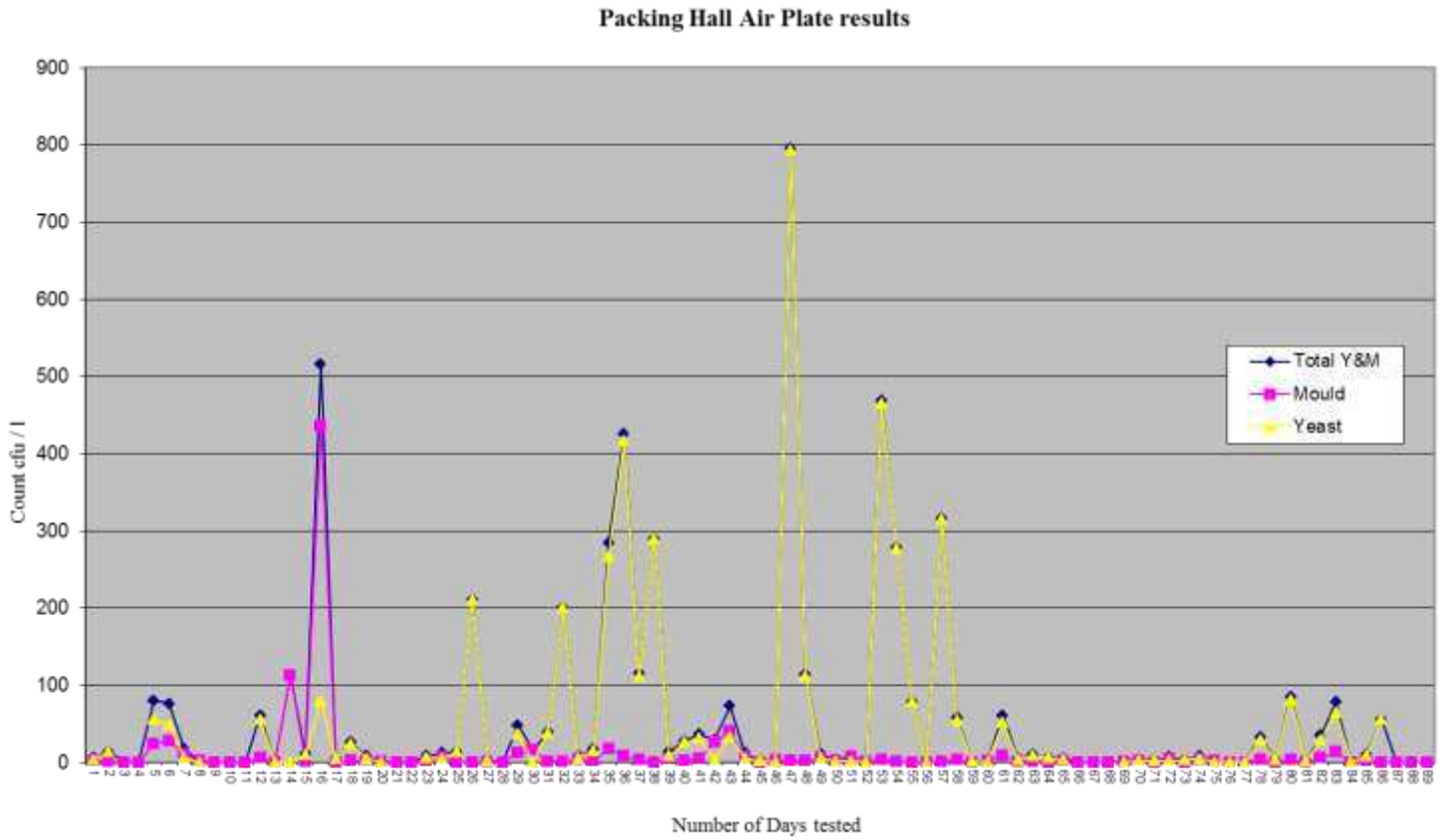


Figure 4.4 Summary of environment exposure plates in the Packing Hall. X axis denotes the test numbers carried out each day over a 3 month period.

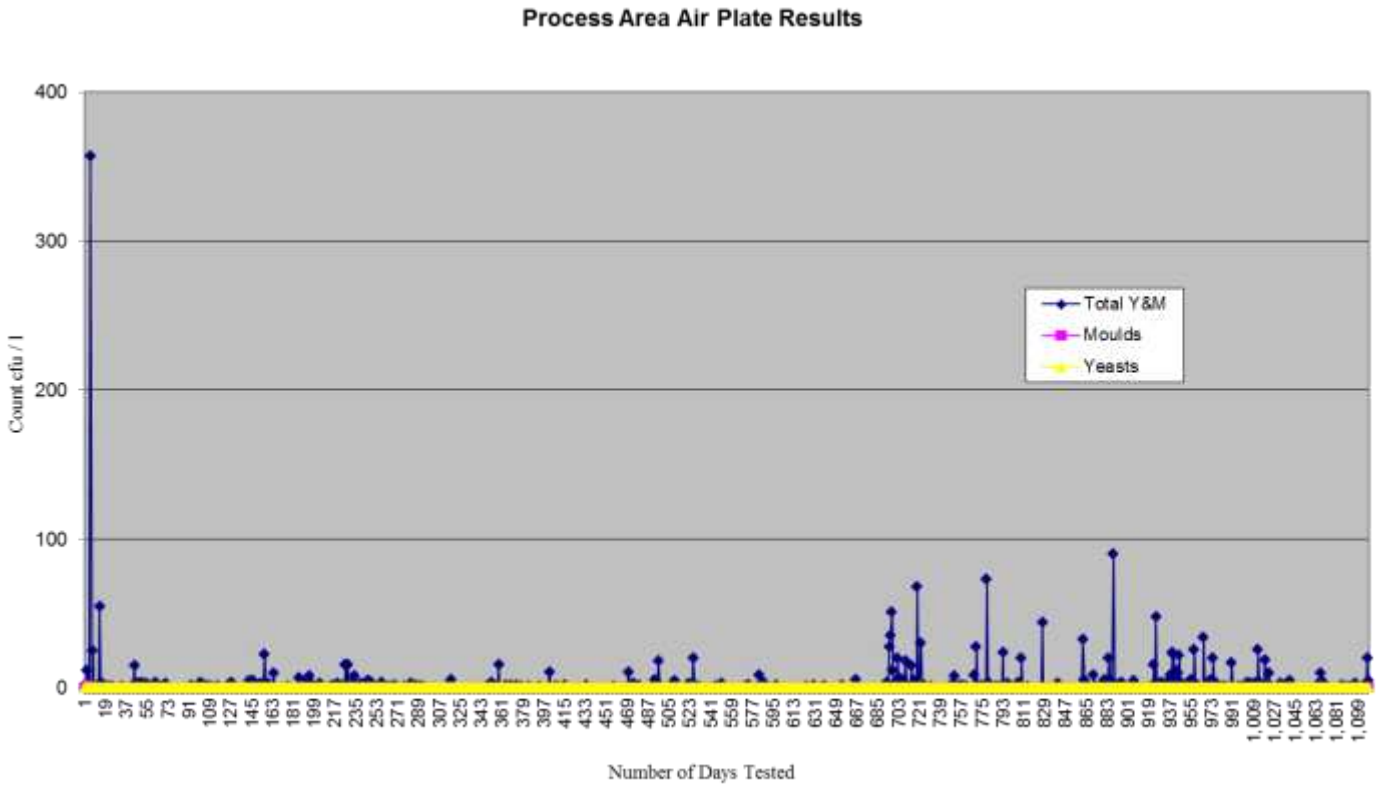


Figure 4.5 Summary of environment exposure plates in the Process hall, Butter making Room and Benhill Area. X axis denotes the test numbers carried out each day over a 3 month period.

When analysing the air samples data (Figures 4.4 and 4.5), it was also observed that the contamination level was higher on the first 24-hour production run in the packing hall area (Figure 4.4) compared to the processing hall. The counts in the packing were as high as 800 cfu/l for yeasts and 436 cfu/l for moulds in the packing hall whereas the levels of yeast counts were not at a detectable level in the process area and only on one occasion mould counts of 357 cfu/l were observed.

Based on the results in Figures 4.4 and 4.5, an air purifier BAXX was installed in the factory near the process and Benhill packing area and the air was sampled for a further 15 months at similar intervals and a significant level of reduction of the contamination was observed (Figures 4.6 and 4.7).

An average count of 2 cfu / l was observed in the packing hall area compared to a level of ‘not detected ‘in the processing hall area, significantly and noticeably less than the counts as high as 800 cfu/ l for yeasts and 232 cfu/ l for moulds observed before the BAXX installation (Figures 4.4 and 4.5).

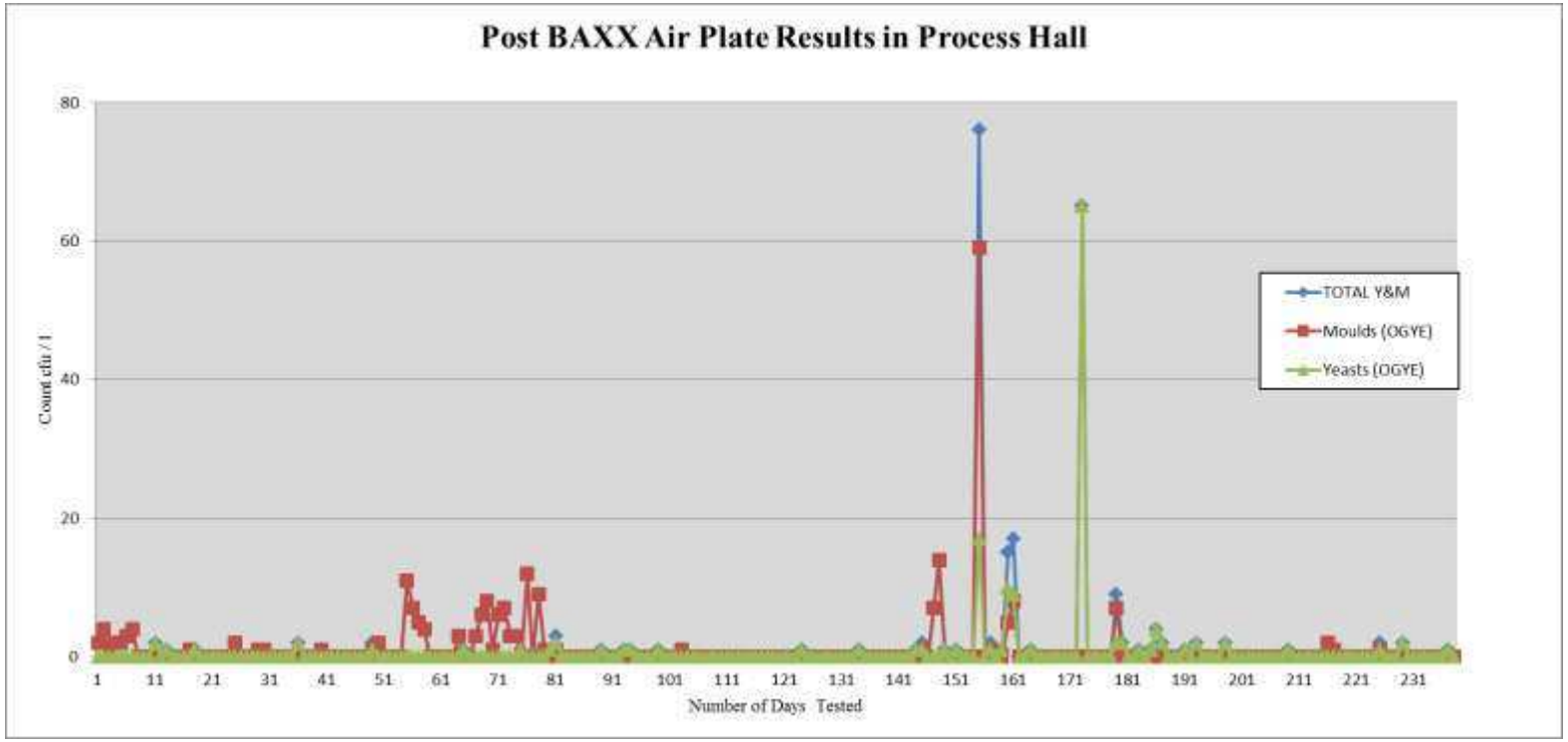


Figure 4.6 Summary of environment exposure plates in the Process Hall post Baxx installation. X axis denotes the test numbers carried out each day over a 3 month period.

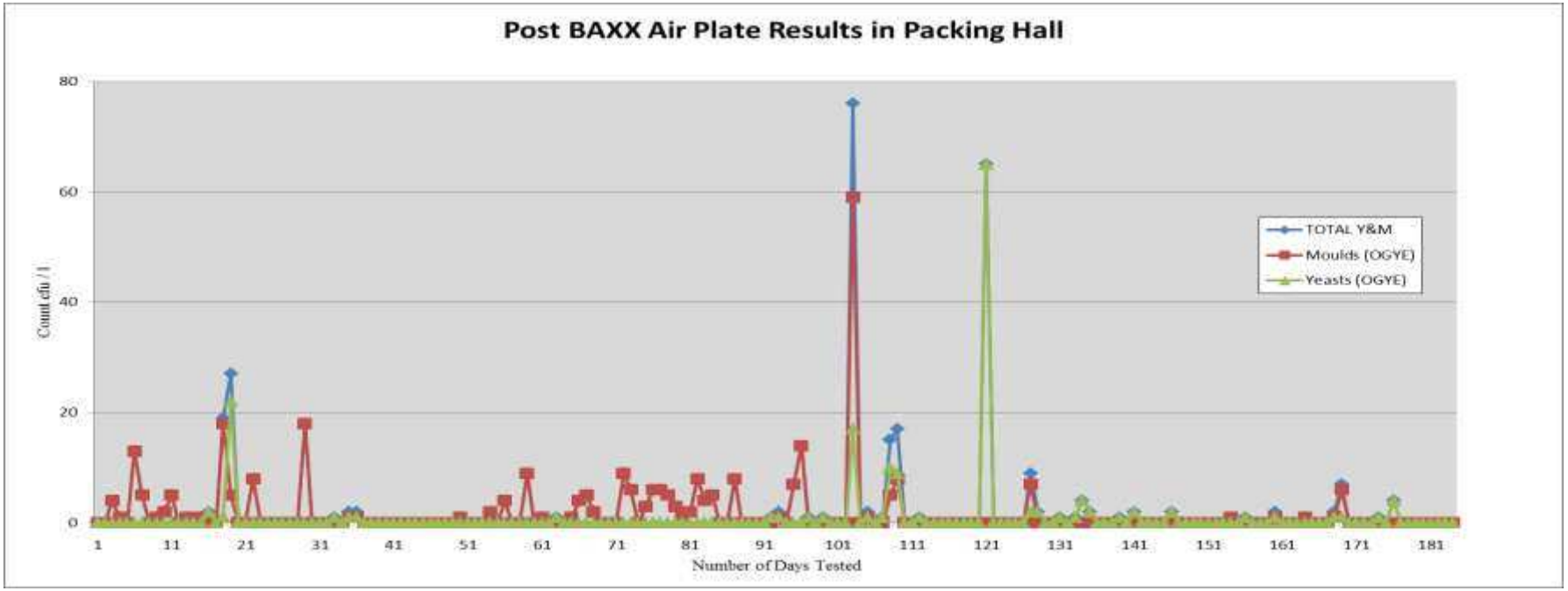


Figure 4.7 Summary of environment exposure plates in the Packing Hall post Baxx installation. X axis denotes the test numbers carried out each day over a 3 month period.

The Benhill area is the packing area for 250 g, 500 g, 1kg and 2kg spread tubs. Packaging alongside cardboard boxes is stored near this area as the tubs, lids and membrane films come in cardboard boxes. These were one of the areas with a high prevalence of yeast and moulds during production time and also just after cleaning. Two BAXX units were installed in this area as a result of this, where there are five filling machines. The results for air sampling in this area are shown in Figures 4.8 and 4.9 showing that the general environment microbial load (TVC) was ≤ 100 cfu/l and for Y&M ≥ 98 % pass rate was observed.

To understand the level of general microbial load in the air, TVC (§3.1.2 and §3.1.4.1) were also performed over a two month period post BAXX installation along with Y&M (Figure 4.8 and 4.9). The pass level for TVC in both packing and process area were ≤100 cfu/l, were most of the results were well within the limit with a failure in five occasions, but when the test was repeated they came back within specification. On the other hand the level of Y&M was reduced from ≤100 cfu/l to ≤50 cfu/l and monitored for six months and 98% of the results were within ≤ 50 cfu/ l with 100% within ≤100 cfu/l.

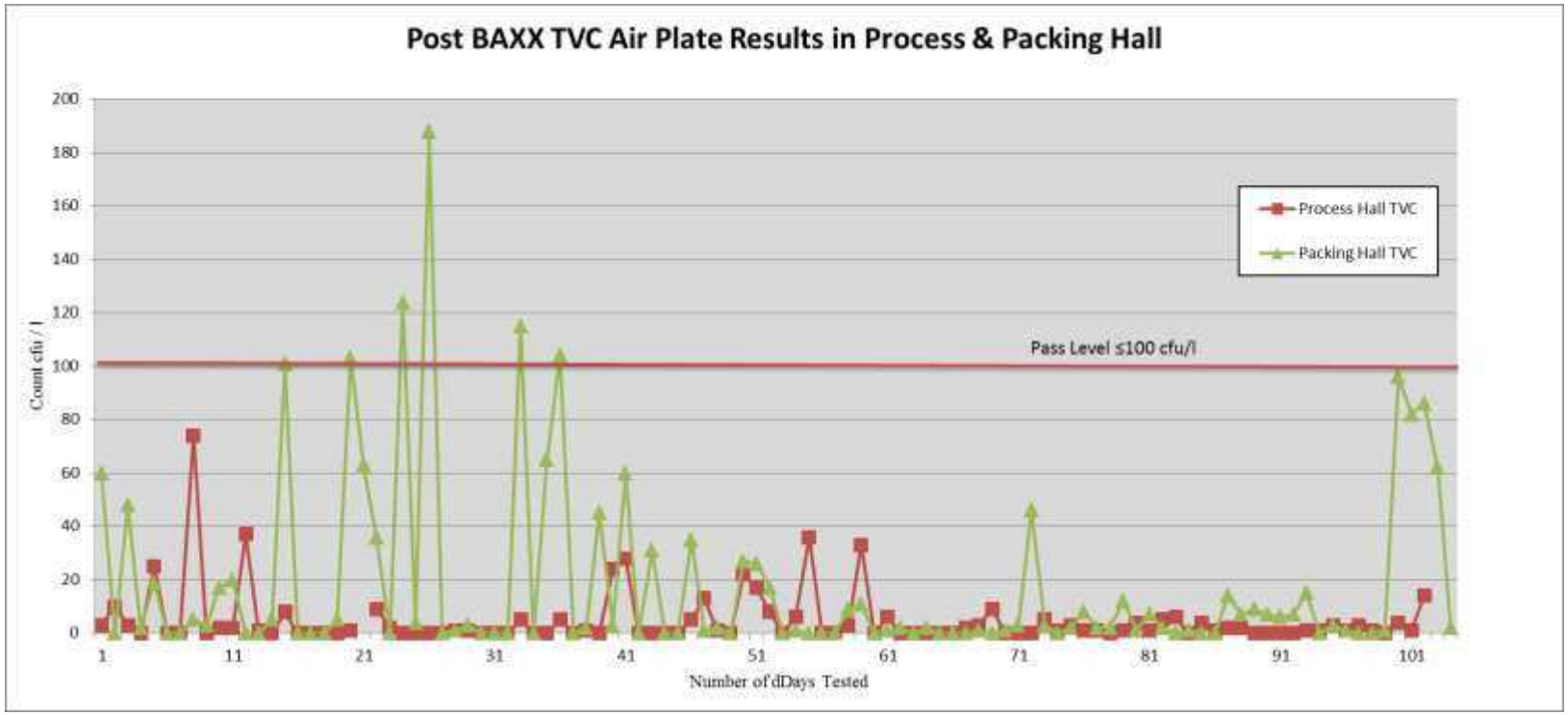


Figure 4.8 Summary of environment exposure plates for TVC in the Process and Packing Hall post Baxx installation. X axis denotes the test numbers carried out each day over a 3 month period.

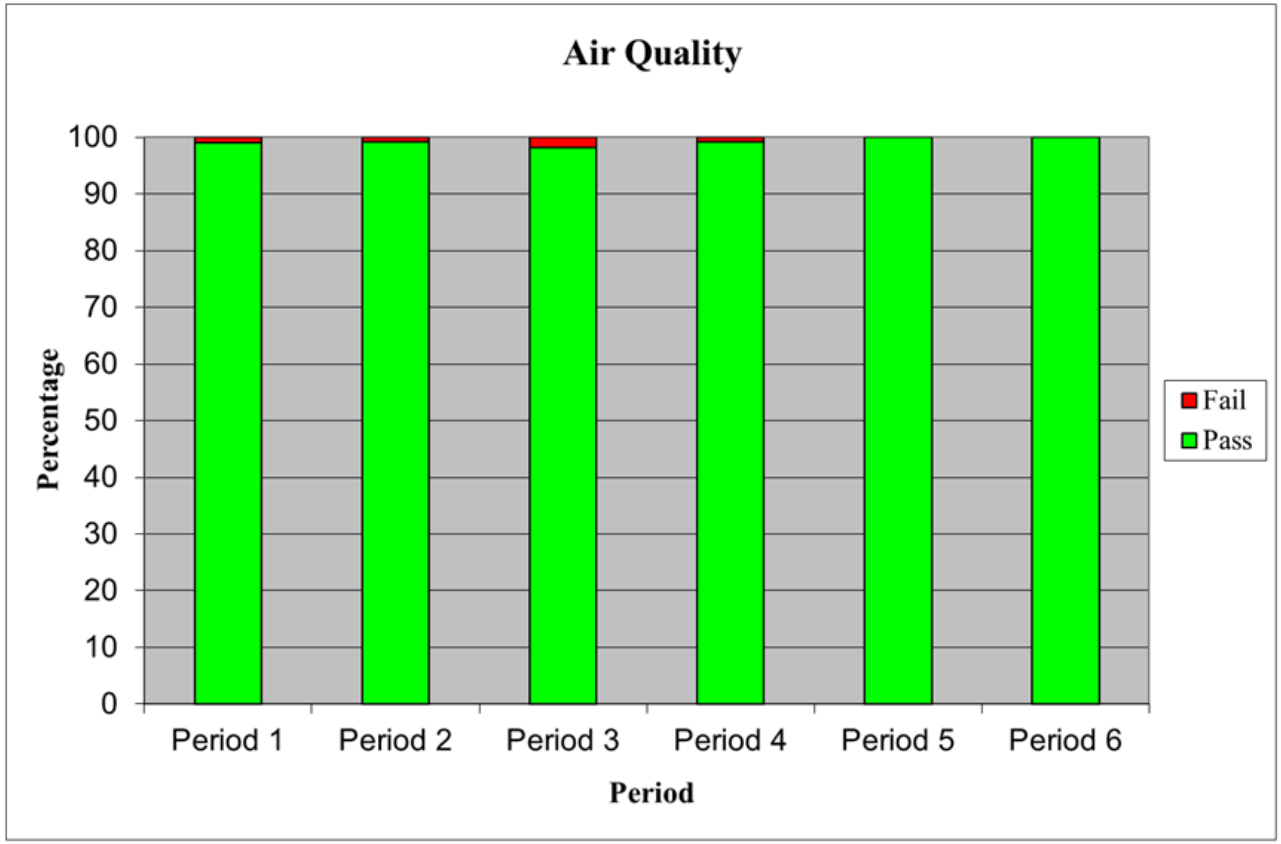


Figure 4.9 Summary of air plate results for Y&M post BAXX Installation of 6 periods (Financial Months) in the Process and Packing Hall where the specification has been reduced to 50 cfu / l from 100 cfu/l.

4.4.3 Water sampling

The bore hole and the general factory water were analysed (§3.1.4.2) for the same microbial contaminants as in the products, ingredients and air (§3.1.2 and §3.1.3). But the analysis showed the absence of all the microorganisms with an occasional count of Enterobacteriaceae which was on a par with the water specification for the production site (Data not presented).

4.4.4 Packaging sampling

The primary packaging of the product such as the tubs, lids and coverleaf were also screened (§3.1.4.3) for the isolates on the 250g and 500g tubs. The tubs and lids were made of polypropylene copolymers and the coverleaf was made of grease proof paper. During the investigation, 50 tubs, lids and cover leaves (25 for 500g and 25 samples for 250g) were screened daily for three months for Bacillus spp, Staphylococcus spp, yeasts and moulds and no contamination was found (data not presented).

4.4.5 Personnel Screening

Personnel with direct food contact in the process and filling area were screened for the presence of Enterobacteriaceae and Staphylococcus spp (3.1.4..4) to prevent post process contamination. Out of the 10 direct food handlers 8 people were positive with the presence of Staphylococcus spp in their hand working at the churn and filling area. The data is not presented here due to confidential reason.

4.5 DISCUSSION

Milk is a complex biological fluid and a very good medium supporting the growth of many microorganisms. Bacterial contamination of raw milk can originate from air, milking equipment, feed, soil and grass (Coorevits et al. 2008). The conditions during storage and transport in refrigerated tanks cause the raw milk microbiota to change from predominantly Gram-positive to predominantly Gram-negative organisms as they grow along with other genera such as Bacillus spp.

Pasteurization of raw milk is effective in eliminating all microorganisms but the thermoduric microorganisms of the genera Microbacterium, Micrococcus, Streptococcus, Lactobacillus, Bacillus, Clostridium, the coryneforms, and occasionally some Gram-negative rods (Jay, 1996) can survive the heat treatment process. Pasteurization cannot guarantee the absence of microorganisms, when they are present in large numbers in raw milk or due to post-pasteurization contamination. (Salmeron et al. 2002). The number of spores or level of contaminant is season dependent, with the highest contamination occurring during the grazing period (Meer et al. 1991, Te Giffel et al. 1995). The samples collected in this study both fresh and complaint samples were produced between February to October covering the harvesting season. The study was not extended further to cover other months of the year due to the research time limitation and to move forward to solve the spoilage issues within the business.

Cream is a bi-product to milk processing and used as an ingredient for spread production, hence it is pasteurised prior to be blended for spread production. The water phase and oil phase are heated again at 83ºC for spread production. A certain number of organisms will survive most processes as pasteurisation is used for cream to make spreads but the levels are usually low as pasteurisation gives a 3 to 4 log reduction in counts (Juffs and Deeth 2007). High numbers may indicate either poor raw product quality or poor hygienic practice during manufacture.

Most of the thermoduric bacteria produce extracellular proteolytic and lipolytic enzymes that are secreted into the milk, which are not inactivated by pasteurizing at 72 °C for 25 s (Griffiths et al. 1981). The residual activities of these enzymes can reduce the organoleptic quality and shelf life of processed milk products (Fairbairn et al. 1986). The ingredients in the spread production are heat treated at a temperature above pasteurisation, it is highly unlikely for any microbes to be present unless they are bacterial spores that can survive the heat treatment (Rarier and Boor 2009) and can also survive in the storage tank for a long time hence Bacillus spp biofilm can form in the processing equipment's. It is known in the dairy industry that during the farming season the raw milk has high prevalence of Bacillus spp (Sutherland 1993). Figure 4.10 illustrates the point of sources from which lipolytic organisms can be introduced into the spread process ((Allen and Hamilton 1994; McKenna 2003).

Spreads exist as emulsions at some stage during the production (Dickinson 1989; Friberg and Yang 1998, McKenna 2003). The overall qualities of an emulsion-

based product are dependent on a combination of physiochemical and sensory characteristics and also exhibit a great diversity of rheological characteristics in terms of viscosity. The raw materials such as the water, oil, emulsifiers, thickening agent, minerals, acids, vitamins, flavours and processing conditions (mixing, homogenization, pasteurisation etc.) determines specific quality attributes of a particular product. The spoilage spread 'A' consists of 75% of fat with fresh buttermilk and vegetable oil, which appears as butter-like, with the colour and texture of butter. It also contains natural colour, flavouring, is virtually trans-fat free and has no hydrogenated oils and half the saturated fat of butter. The product is suitable for spreading, cooking, frying and freezing. It contains a minimum of 21% moisture and 1.5% salt. The finished product is stored and distributed at 5ºC with 90 days shelf life.

Figure 4.11 shows the study outline for the microbiology analysis. The study was divided into three main sections, finished product, ingredients and post process contaminants as people, air etc. The date range for the complaints samples varied from 'Best Before' April to October 2009 with a total shelf life of 12 weeks. All the samples were tested at the end of their shelf life. Higher percentages of complaints were observed in June – July due to the marketing promotions, where consumers bought multiple products and stored them under refrigeration or freezing conditions. The fresh samples were products produced in October 2009 and all the tests were carried out at the same time. Return samples from the consumers between April to September were stored under frozen temperature to stop further spoilage. As per this section the bacterial isolates from both product

and environment were Staphylococcus equorum, Staphylococcus pasteuri, Staphylococcus sciuri, Bacillus subtilis and Bacillus licheniformis. In the API STAPH S warneri was identified. S pasteuri could be identified as S. warneri in the API STAPH system as they are phenotypically similar but genetically more closely related to each other (Chesneau 1993). The Staphylococcus isolates were from the product handlers in the process room. Y&M isolates from the factory air were Pichia guilliermondii, Candida guilliermondii, Candida parapsilosis and Candida ortho parapsilosis.

High prevalence of the presence of Bacillus spp was noted in the spreads ingredients as buttermilk, skim milk and cream post pasteurisation compared to other isolates as Staphylococcus spp and Y&M. Bacillus spp contaminant was carried through after the pasteurisation. The level of contaminant increased with the age of the ingredients i.e., after 42-hours from the last pasteurisation, the microbial load significantly increased at least by one to two fold. Other ingredients as oil and water were of low risk.

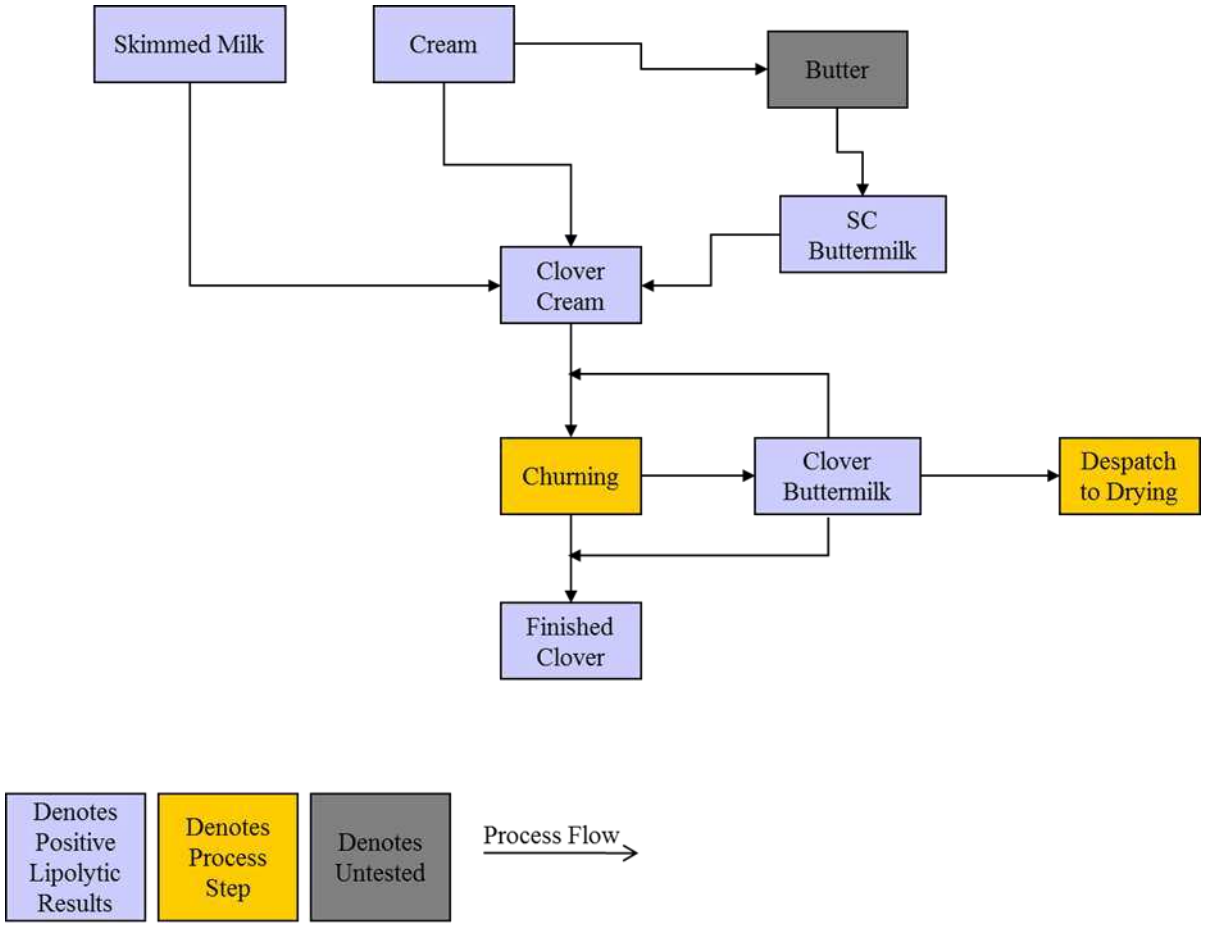


Figure 4.10 Areas of positive lipolytic organisms found in the spread processing (Allen and Hamilton 1994; McKenna 2003).

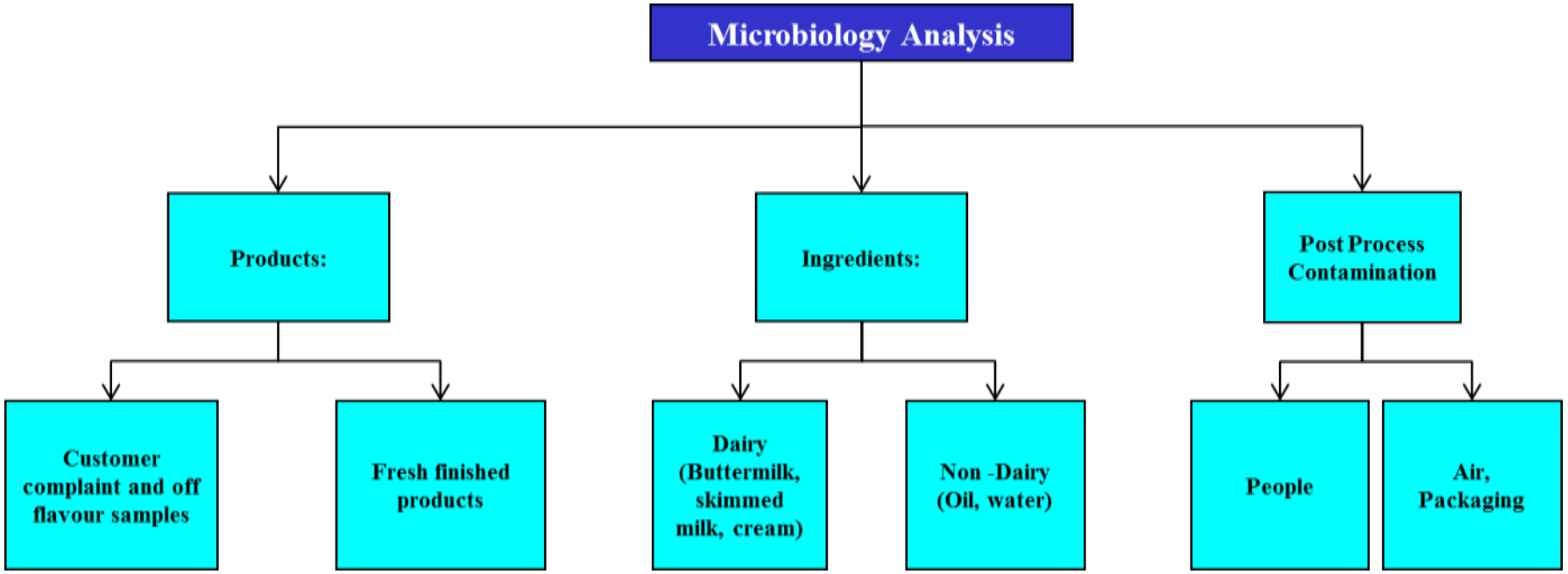


Figure 4.11 Study outline for microbiology analysis

Staphylococcus spp was isolated form personnel at post pasteurisation stages. Good personal hygiene policies and practices are the foundation for successful food safety and quality assurance in all food manufacturing facilities. Plant personnel are among the most significant reservoirs and vectors of microorganisms, chemical residues and foreign materials in the food facility, and as such, can be a source of unwanted contamination to products. Comprehensive personal hygiene programs, coupled with a top-down philosophy supporting sound sanitary practices as part of the corporate structure, are key to implementing best practices for compliance with Good Manufacturing Practices (GMPs), Standard Operating Procedures (SOPs) and related sanitation and food safety audits. The top-down approach ensures that personal hygiene policies and procedures are implemented by all personnel—management, visitors, production, sanitation and maintenance staff—at each company and in each facility, reducing the risk of product contamination and the likelihood that such product will reach the consumer. A regular monthly monitoring is implemented throughout the food processing area, mainly concentrated on people who come in direct contact with the food. Coagulase positive staphylococci (CPS), the group of bacteria to which enterotoxigenic S. aureus belong, can be readily isolated from raw milk sometimes at high concentrations ($>10^6$ CFU /ml) (Andrew 2003). It is generally accepted that the minimum temperature for growth is 7°C, but the minimum temperature for enterotoxin production is 10°C. Hence if the milk is pasteurised properly and the chill chain is held as per hygiene regulation ≤8ºC S. aureus contamination should be absent in processed milk products.

The third source of contaminant from the environment was the processing hall air which is filtered and the pressure is monitored. But the spread site has a moist filling area and it is not filled in aseptic filler. Therefore the product may get contaminated after processing. When analysing the spread samples it was also observed that the contamination level was higher on the first 24-hour production run indicating aerosol contamination rather than cleaning or hygiene issue. Y&M were the only contaminant identified in the air showing lipolytic activity.

An air purifier system BAXX was installed around the factory after the findings. The Baxx system uses novel cold plasma technology to produce a highly active antimicrobial environment and BAXX claims to kill all known airborne and surface-active pathogens including, viruses, moulds, bacteria and spores (BAXX user manual 2010). BAXX proprietary patented process works on a discharge based non-thermal plasma ion multiplier. A burst of high energy electron (1`10eV) is created in an ambient environment consisting of air, water moisture and pollutant where the electrons collide with the dominant background molecules, creating highly active radicals reacting against bacteria, virus and Y&M without the use of toxic compounds within a food factory. The hydroxy1 radical removes a hydrogen atom from one of the carbon atoms in the bacteria cell structure to form a molecule of water thus disrupting the cell. Oxygen free radicals also initiate oxidation in the cellular, mitochondrial, and nuclear and endoplasmic reticulum membranes and increases the cellular permeability for Ca^{2+} and increased cellular concentrations of calcium ions damages the mitochondria of the bacteria. Amino acids are oxidised and degraded. Nuclear and mitochondrial DNA is oxidised, resulting in strand breaks and other types of DNA

damage to the bacteria. As a result, bacteria, virus and moulds are disintegrated and die off. The unit has shown an effectively kill on Escherichia coli, Staphylococcus aureus, Campylobacter spp, Listeria monocytogenes, Pseudomonas spp and general Y&M populations, this was validated by the BAXX providers by performing a challenge test at Campden (data not presented).

The factory air was sampled for a further 15 months in similar intervals after the installation of the two BAXX units across the production hall near the five filling machines. It was shown that significant reductions of the contaminants were observed. The results for air sampling showed that the general environment microbial load (TVC) was ≤ 100 cfu/l, which aligns with GMP and for Y& M a 100% pass rate was observed.

As a part of HACCP (Hazard Analysis Critical Control Point), the packaging suppliers are a part of a pre-requisite programme that has a specification of absence of Bacillus spp and yeasts and moulds in order to comply with the finished product specification. The factory as a part of their Quality Monitoring Plan verify the packaging monthly and no issues were noted from the time of the 'cheese flavour' samples received (§4.4.4) indicating the main source of the contaminates were the raw materials age over 24 h old, factory personnel with direct food contact and the factory air.

CHAPTER 5 CHEMICAL ANALYSIS

5.1 RHEOLOGY

Rheology was measured to check compliance with product specification or as part of an investigation into the effects of changes in process parameters and to assess the consequences of changes in ingredient formulations.

The test was carried out on 300 different tubs (150 'cheese flavour' samples were compared with 150 standard products) each in duplicate across 3 layers (1= top layer, 2 = middle layer and 3 = bottom layer) of the spread. They were allowed to equilibrate in either a waterbath at 20ºC or in a storage area at room temperature for at least 1 hour, but preferably overnight in order to equilibrate them to the same temperature as per method 3.2.1.1. The samples were carefully divided using a sterile wooden spatula to avoid any extra aeration. Each layer was divided into two further Sections to take an average firmness of each layer. From Figure 5.1 it was noticed that the top layer was significantly softer in the 'cheese flavour' products to that of the standards. The mean firmness value for the top layer product distribution of the spread samples were 10% lower in the 'cheese flavour' tubs, with subsequent layers being much the same across all samples as the middle and bottom layer rheology was consistent.

The results generated (300 tests) indicated that there is a significant variability in the rheology within a tub. Variability within each layer was generally similar

(standard deviation ~ 14%) with the exception of the top layer of the 'cheese flavour' tubs, which would indicate increased product heterogeneity at the surface (Figure 5.1).

5.2 FREE FATTY ACID ANALYSIS (FFA)

GC-MS method as per Section 3.2 was used to analyse 'cheese flavour' samples for the presence of methyl ketones, as this would help to identify the source of the strong 'cheese flavour' cheese smell. The results are summarized in Table 5.1. It was observed that the complaint samples contained free fatty acid (FFA) C10 and C12 that gives the cheesy fruity floral smell. Out of the total 300 'cheese flavour' samples analysed, 62% contained C10, 52% C12 and 32% had C8, compared to only 7% with C6. Also, octanoic, decanoic, dodecanoic, tetradecanoic (C14) and hexadecanoic (C16) were present showing the breakdown of triglycerides.

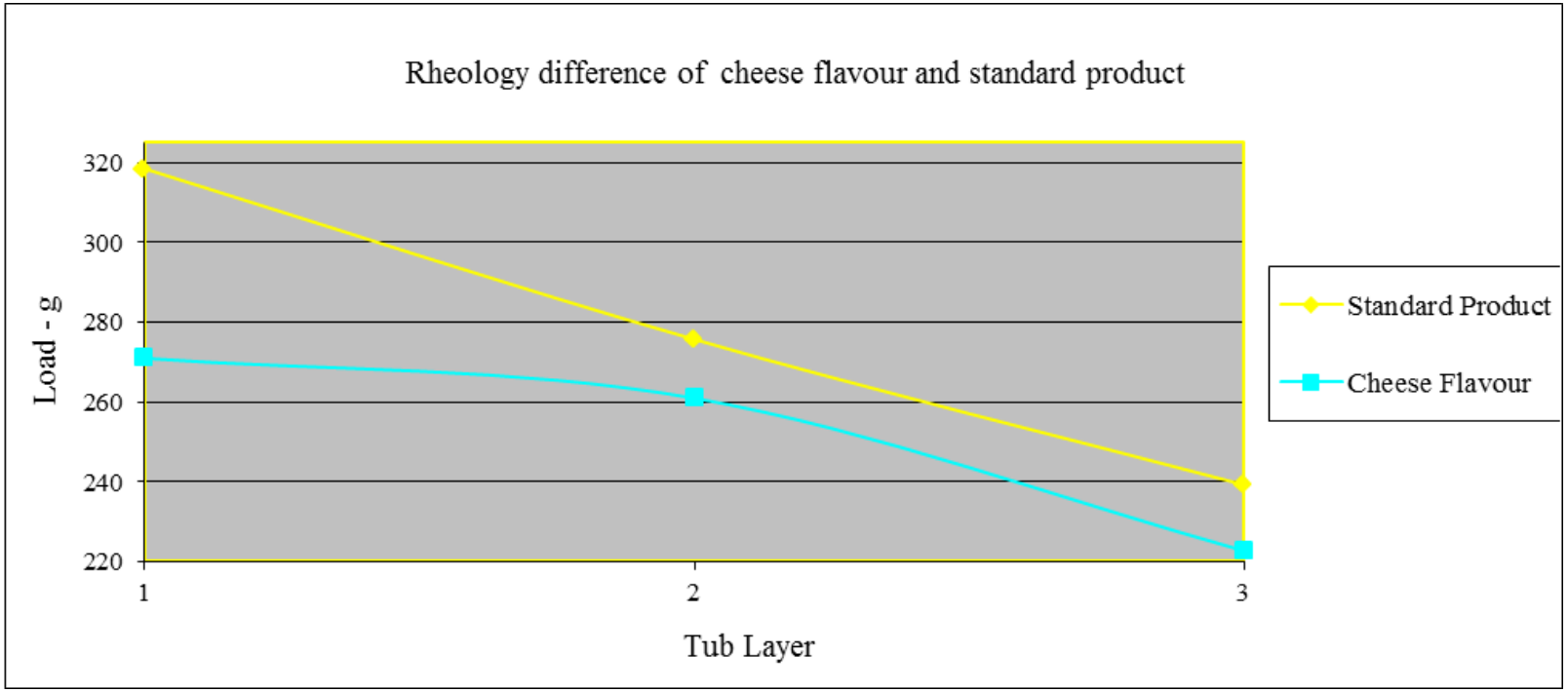


Figure 5.1 Difference in rheology between the 'cheese flavour' and standard product

((1= top layer, 2 = middle layer and 3= bottom layer). Each layer was tested twice and an average was taken)

5.2.1 FFA profile compared against other market spreads

To benchmark the spread under investigation against other market spreads, methyl ketone was analysed in 10 different other competitor companies' spreads (Table 5.2). Spread A, A Lighter, B, C, C Lighter, D are produced by UK market whereas H, I, I omega 3 are produced by EU manufacturer and the rest of the products were similar competitor products available in the market (E, F butter, F Spread, F unsalted, F slightly salted, G and G light and J). The standard product samples were A and C. The A light and C light products have salt concentration reduced to 1.5% from 1.7% (Table 5.3). A is a product with slightly different formulation and processing conditions to the C at another manufacturing site.

It was observed from Figure 5.2 that the standard UK products (Spread A, A Lighter, B, C, C Lighter, D) were unusually high in C12 compared with the general market place with the EU spread samples (H, I, I omega 3) being the highest. This coupled with the fact that 42% of the generated methyl ketones are derived from C12 formulated the hypothesis that the fat phase containing C12 was the principal component being utilised by the causative organisms. 'J' was only 14 days old in a 90 days shelf life and had a very slight hint of 'cheese flavour' smell with a percentage of presence of C12 at just above 4% wt. of the total extracted fat (Figure 5.2). Therefore it is expected that the level of C12 would increase with life and spoilage. In most of the market spreads, extracted C12 ranged from 0 to a maximum 4% weight compared to an average of greater than 9% in the company spreads A, A lighter and B.

5.2.2 Sensory analysis compared against competitor products

A total of 100 samples were analysed for sensory attributes as per method 3.2.1.9 of which 50 were of 'cheese flavour' samples and 50 fresh standard products. As the 'cheese flavour' samples were taken from the quality rejection sample storage area, they were all at the end of their shelf life (100 days; 77 days declared life and extra 15% for GMP). The standard products were freshly produced off the line and tempered. The analysis was performed at two different temperatures, 5ºC and 20ºC to analyse the effect of temperature on the rate of spoilage. Again 10 different other market products were tested alongside.

For the standards and the other market products the spreads were tested three times during the shelf life of the product, at the start, middle and end of life. The samples at the start of life were kept at 20ºC for 4 hours to mimic the general consumer and chill chain abuse in the supply chain. In order to aid with the sensory test, a 'cheese flavour' meter was developed as per Table 5.4.

Table 5.1 Principal methyl ketones analysis by GC-MS

Methyl Ketone	Principal FFA	FFA Carbon Chain Length	% +ve in 'Cheese Flavour' Samples	Associated Sensory Descriptor
2-pentanone	Hexanoic acid	6	7	Acetone, fruity
2-heptanone	Octanoic acid	8	32	Cheese flavour cheese, fruity
2-nonanone	Decanoic acid	10	62	Cheese flavour' cheese, fruity, floral
2-undecanone	Dodecanoic acid	12	52	Citrus, floral

Table 5.2 Summary of various spreads used in the challenge test

Spread Name	Country of Origin	Company	Fat %
A	UK	Own (Factory A)	80
A Lighter	UK	Own (Factory A)	50
B	UK	Own (Factory A)	67
C	UK	Own (Factory B)	75
C Lighter	UK	Own (Factory B)	55
D	UK	Own (Factory B)	65
E	UK	Competitor	78
F Butter	EU	Competitor	80
F Spread	EU	Competitor	78
F Unsalted Spread	EU	Competitor	78
F Slightly Salted Spread	EU	Competitor	57
G	UK	Competitor	70
G light	UK	Competitor	30
H	EU	Own (France)	80
I	EU	Own (France)	80
I Omega 3	EU	Own (France)	75
J	EU	Competitor	70

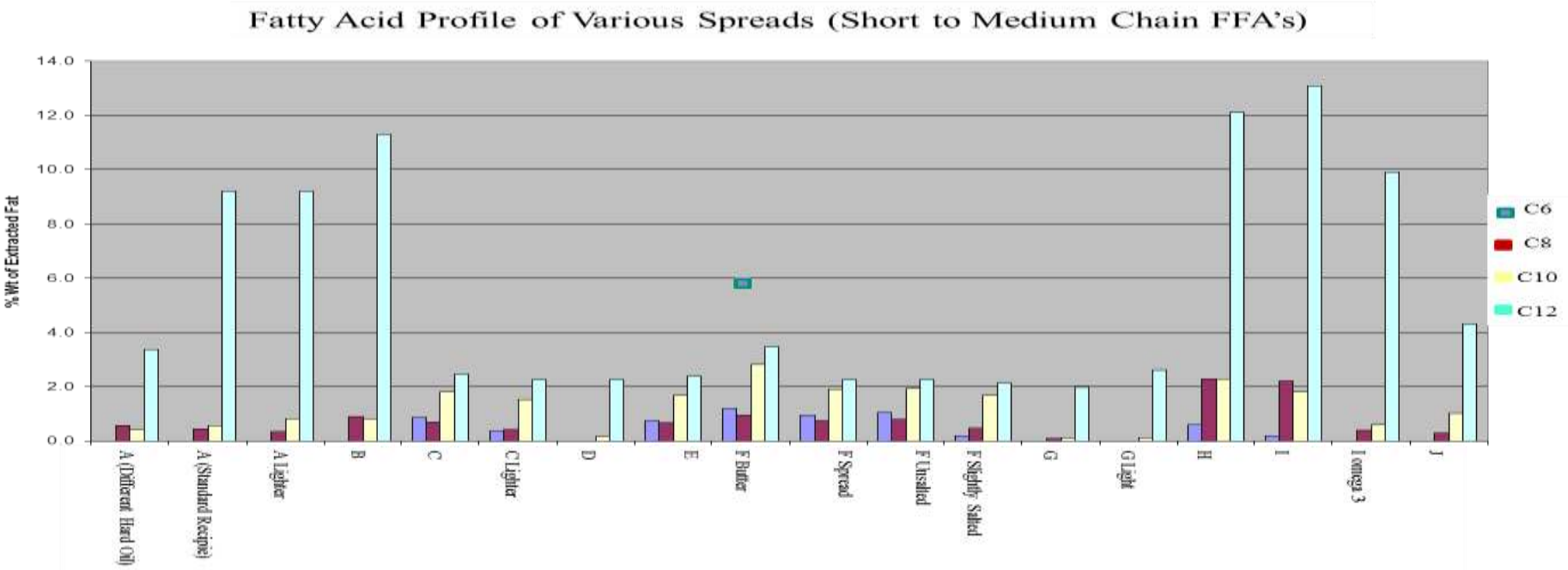


Figure 5.2 FFA profile of company against other market products.

Spread A, A Lighter, B, C, C Lighter, D are produced by UK market and H, I, I omega 3 are produced by EU market and the rest are competitor products (E, F butter, F Spread, F unsalted, F slightly salted, G and G light)

The results were summarised in Appendix 10.5 Table 10.2. It was observed that none of the standard samples throughout their entire shelf life showed any sign of ‘cheese flavour’’ aroma or product breakdown when stored and tested at 5ºC. At 20ºC 28% of the standard freshly produced samples had a faint ‘cheese flavour’ smell with no signs of microbial spoilage (B1) at the start of life (Day 7) after testing at 20ºC. The number of positives at 20ºC increased slightly to 30% within 50 days of the total 100 days shelf life. At the end of the product life (100 days), 36% of the samples at 20ºC showed either ‘B1’ or ‘B2’ signs. This shows that the percentage increased from 28% to 36% over the 100 day shelf life indicating it is highly unlikely to be microbial contamination as spoilage microbes can grow at refrigerated temperature (Jay 1996). The slight increase of ‘cheese flavour’ may be due to the factor of a high concentration of C12 being present in the product formulation in the first place.

On the other hand, 72% of the ‘cheese flavour’ samples were scored under the ‘B1’ or ‘B2’ category at 5ºC. A few ‘B3’ scores were also observed. But the number increased to nearly 90% positive when evaluated at 20ºC. Most of the samples scored as ‘B2’ became ‘B3’ and a lot of the ‘ND’ samples started showing signs of ‘cheese flavour’ aroma as well.

Out of the 10 market spread samples, none showed any sign of ‘cheese flavour’’ from the start to end of shelf life at either 5ºC or 20ºC.

5.2.3 Free fatty acid (FFA) profile in oil

As the ratios of the MK (C12) formation in ‘cheese flavour’ samples were relatively higher (52%) as per Table 5.1 and an increase taint was observed in the sensory testing of the standard products, it may be possible that the oil was one of the main source for the development of ‘cheese flavour’ taint. However, the percentage of ketones formed will also be dependent on lipase specificity.

The spread formulation oil blend was analysed for FFA (method 3.2.1.2) along with standard milk fat for a comparison in the level of C12 generation. In Table 5.4, it is clearly noted that the oils have a higher percentage of C12 (Dodecanoic Acid), 17.4 % compared to only 3.5% in the milk fat which corresponds with the quality taint ‘cheese flavour’ products and also give a higher percentage of ‘cheese flavour’ ketones (52%, Table 5.1).

Table 5.3 Spread formulation (A, A Light, B, C, C Light and D)

Ingredients / Compounds	% of ingredient					
	Spread A	Spread A Light	Spread B	Spread C	Spread C Light	Spread D
Water	29.02	15.16	16.62	12.4	64.4	38.5
Salt	1.7	1.5	1.7	1.5	1.3	1.6
Vegetable Oil	64.25	78.53	76.8	27	27	16.04
Starch	2.0	3.12	-	-	-	-
Buttermilk Powder	3.31	1.57	2.9	-	22.2	1.0
Potassium Sorbate	-	0.063	-	-	-	0.125
Lactic Acid	-	0.07	-	-	-	0.018

Table 5.4 Cheese flavour meter for the sensory test

Cheese Flavour Meter	Category Details
ND	No smell or no visible sign of mould growth or product breakdown
B1	Faint 'cheese flvaour' but no visible sign of mould growth or product breakdown
B2	Strong 'cheese flvaour' but no visible sign of mould growth or product breakdown
B3	Strong 'cheese flvaour' with visible signs of mould growth or product breakdown

Table 5.5 FFA profile of the ingredients in the product

FFA	Methyl Ketone	% Wt. (FFA) in Milk Fat	% Wt. (FFA) in Oil Blend
Hexanoic Acid	2-Pentanone	1.4	0.1
Octanoic Acid	2-Heptanone	1.7	1.3
Decanoic Acid	2-Nonanone	2.6	1.3
Dodecanoic Acid	2-Undecanone	3.5	17.4

5.3 PRODUCT HURDLE RATE DETERMINATION OF SPREADS

Within food matrices the robustness of the food to prevent microbial growth can be difficult to predict as this depends on a variety of factors that all combine to limit growth. In simple terms each growth inhibitor can be viewed as a 'hurdle' that the microbes have to overcome to grow. Hurdles are rarely simply additives as synergistic relationships occur where the linking of one hurdle to another results in an overall increase of the growth inhibition which is greater than some of the sum of the parts. On the basis of the findings so far in Sections 4 and 5, the standard and key competitor samples were further analysed for the hurdles important in spread microbial stability.

The hurdle rates were determined in an array of spread sample with the manufacturing company and the competitors in the market. Standard spread (A, A Lighter, B, C, C Lighter and D in the UK market and competitor spreads E, F Butter, F Salted, F Unsalted, F Light, G and G Light were used to standardise the hurdle rate of the affected product along with other verities produced by the manufacturer.

5.3.1 Water droplet size

Shells of fat crystals covering water droplets prevent these from merging together (Juriaanse and Heertje 1988). The largest hurdle within yellow fat technology is the size of the moisture droplets distributed within the product. To sustain

microbial population the accepted literature value for minimum droplet size is ≥ 5 µ in radius (Brocklehurst 1995) but to allow growth it requires to be ≥ 10 µm. Currently the operation site works to a droplet size target of 95% ≤ 10 µm.

A comparison of the moisture droplet distribution was performed in standard spread formulation (A, A Lighter, B, C, C Lighter and D) and competitor spreads (E, F Butter, F Salted, F Unsalted, F Light, G and G Light) as per method 3.2.16. Standard products 'A' and 'B' have a poorer droplet size distribution and struggle to achieve the 95% ≤ 10 µm (Figure 5.3). More important is the clear difference between some of the standard products 'C' and 'C lighter' achieved 98%≤5 µ level. On the other hand, the majority of the competitors (F Butter, F Salted, F Unsalted, F Light, G and G Light) were achieving 98% ≤ 5 µ other than spread 'E' at the level of 90% ≤ 5 µm, thus leading to inherently more microbial stable products.

In the case of Spread 'A' the droplet size % ≤ 5 µm is significantly lower than the other market competitors at around 68%. This difference in droplet distribution is linked to the process technology utilised to produce the product and specifically the SSHE process. In the case of spread 'C' and 'C lighter' the droplet distribution is good (greater than 98% ≤ 5 µm) as the product is already made by SSHE process compared to the traditional churn process. This in turn leads to a greater degree of microbial stability and hence more robustness against 'cheese flavour'' causative organisms.

5.3.2 pH

The pH of the aqueous phase is a secondary hurdle to microbial growth after droplet size and is usually controlled by the addition of an organic acid such as lactic acid (Charteris 1995). Most bacteria are inhibited by acidic, low pH conditions and for Listeria monocytogenes in particular the minimum pH required for growth at 20 or 30ºC is between 4.3 and 4.6 (Voysey et.al. 2009). Unsalted and slightly salted products generally contain lactic acid for flavour and to make up for the reduction in salt which acts as a microbial inhibitor.

As per Figure 5.4 the pH range of spread A, B, C and C lighter were near neutral (6.5 to 6.8) and are mostly associated with salted product ranging from 1.5 to 1.6 (Table 5.3). On the other hand the A lighter and D had slightly acidic pH of 5.5 with % salt ranging 1.3 and 1.6 respectively (Table 5.3). Most of the competitor products with salt or unsalted had a pH range of 4.7 to 5.2 other than sample E.

Bacterial inhibition at neutral pH is minimal and there is academic evidence that at these levels the oxidation of fatty acids to methyl ketones is optimised (Stead 1986). The disadvantage of reducing pH within the Spread ‘A’ formulation process is that this may cause a significant change to the flavour of the product. The effects of pH and salt levels on the growth of microorganisms are combinations of pH (3.8-5.5) and total salt levels between 1.5- 5% (Holliday et al. 2003).

5.3.3 Concentration of % salt in waterphase

The level of salt dissolved within the aqueous phase of the product (% salt in moisture) is a further hurdle that controls microbial growth (Gomez 1992). Spread 'A' and 'B' were the two spreads with the highest concentration of % salt in the aqueous phase (Figure 5.5) which reflects the need for this hurdle to compensate for the reduction in the hurdle rate afforded by pH. Of the competitor samples analysed most either met or approached closely the 2012 FSA salt range of 1.15 to 1.63 % salt or 450 to 650 mg of Sodium.

5.3.4 Water activity

All microbes require water to multiply and grow. The measure of how much water is physically available for microbial growth is the water activity (aW) of the product and is principally affected by the amount of water within the product and the concentration of solutes such as salt dissolved within that water. Traditionally aW is the principal measure utilised in growth prediction software (Combase), however, aW needs to be considered in the context of the product matrix under review. Within spread, aW gives an overall indication of the susceptibility of the product to microbial growth as the antimicrobial effectiveness of sodium chloride is dependent on the ability to influence the level of water (Charteris 1995).

Taking this into account, standard and competitor spreads were tested for a_W as per Section 3.2.1.7. The results (Figure 5.6) demonstrate that the standard spreads compare well to the general market having the lowest water activity in the cases of Spread 'A' and 'B' due to higher salt % in the formulation (1.7%). But this better hurdle will be significantly offset by the increased droplet sizes (≤90%

<10μ and ≤ 75% in the range of <5 μ) within these products. Whereas spread A, C and C lighter had relatively higher $a_W \geq 0.96$ along with the competitor spreads E, F unsalted, F light, G and G light. F butter and F salted on the other hand had similar range of a_W as A and B spread.

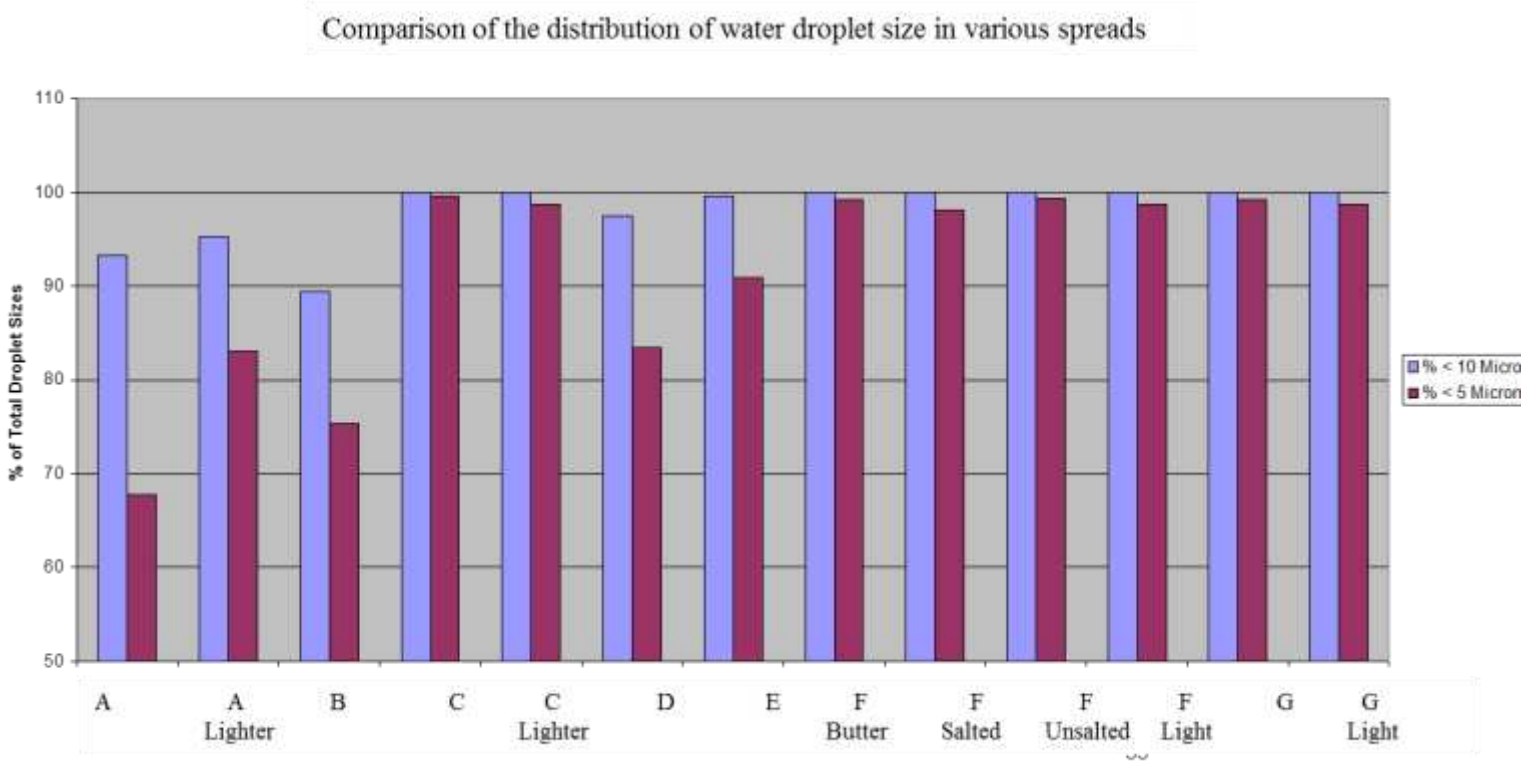


Figure 5.3 Comparison of standard spread formulation (A, A Lighter, B, C, C Lighter and D) and competitor spread (E, F Butter, F Salted, F Unsalted, F Light, G and G Light) droplet sizes.

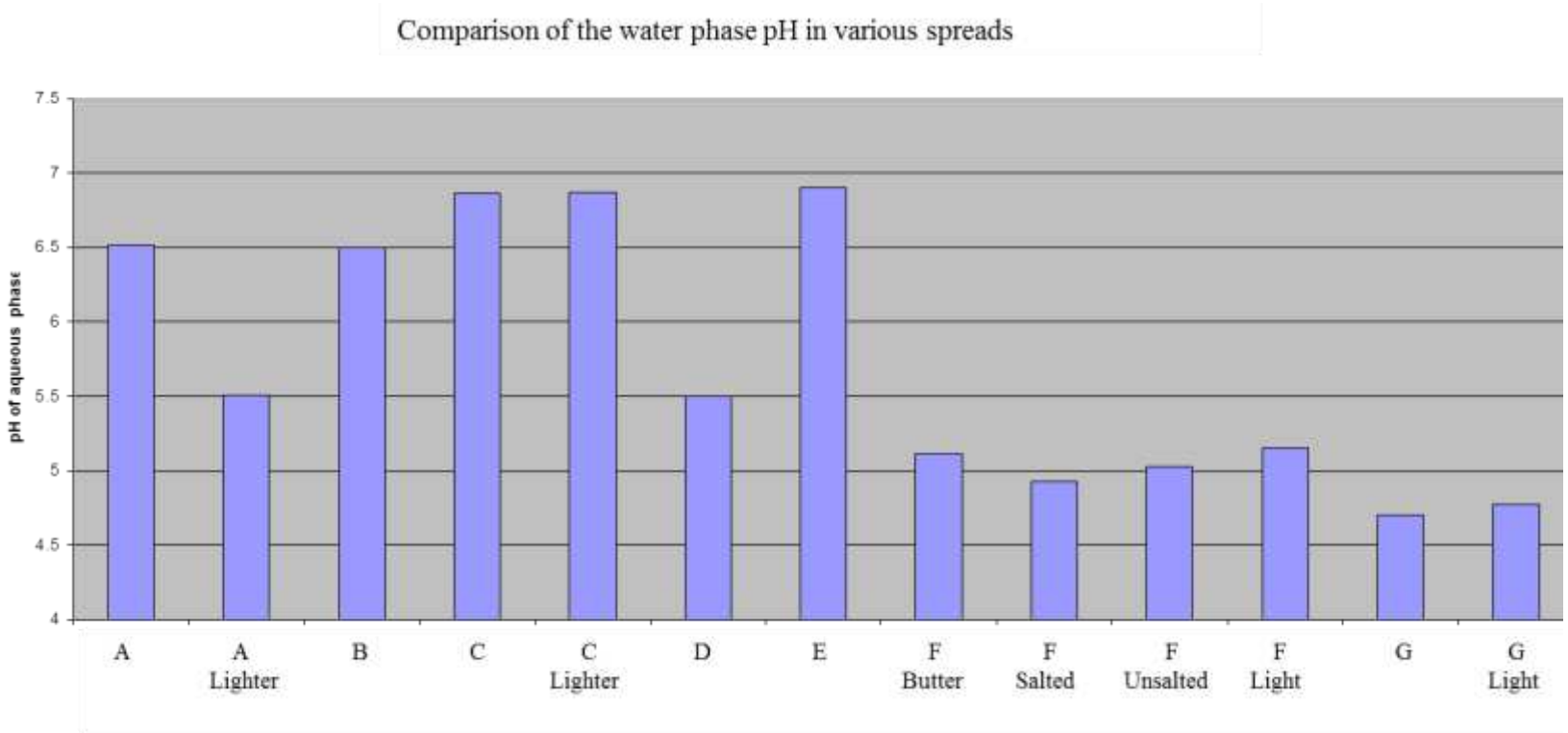


Figure 5.4 Comparison of standard spread formulation (A, A Lighter, B, C, C Lighter and D) and competitor spread (E, F Butter, F Salted, F Unsalted, F Light, G and G Light) pH in the aqueous base.

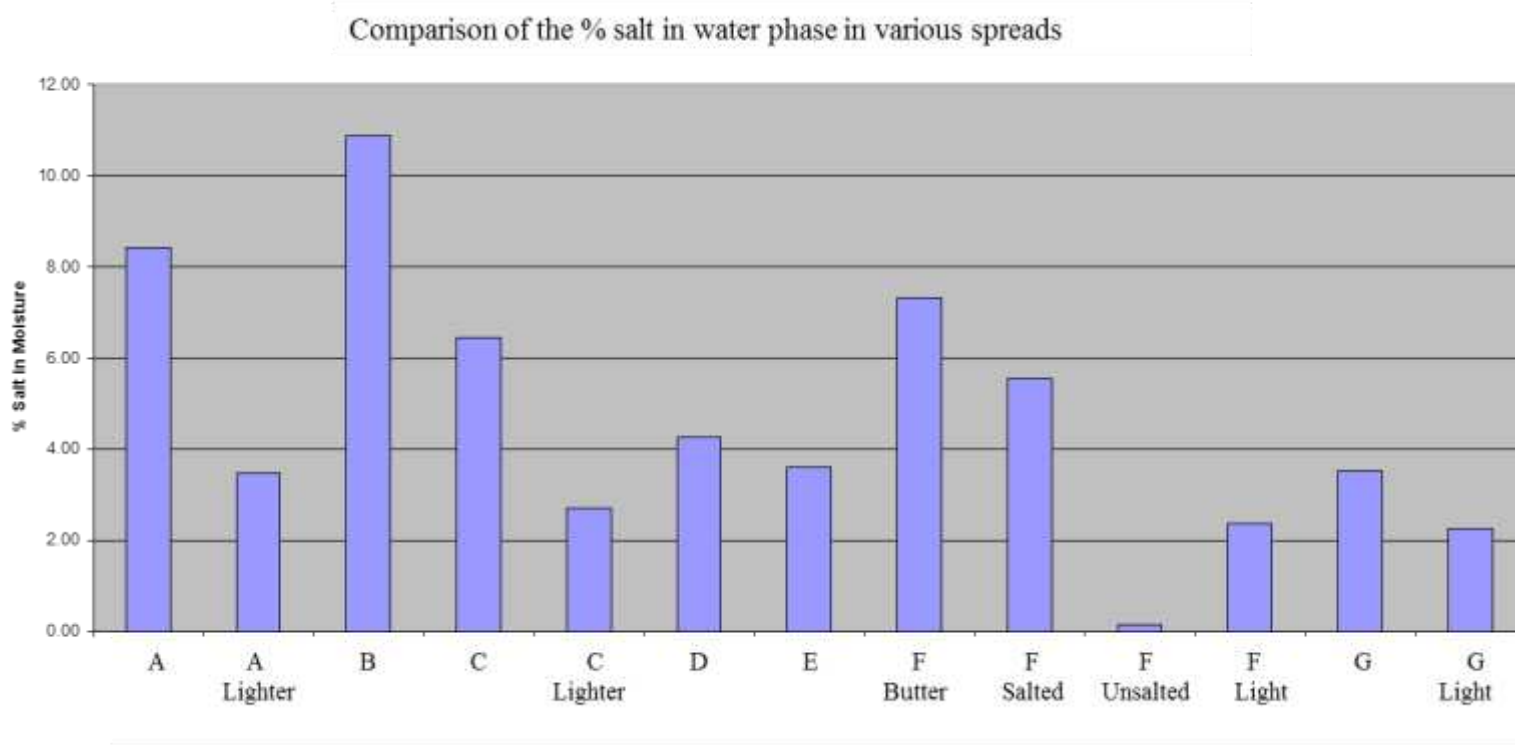


Figure 5.5 Comparison of standard spread formulation (A, A Lighter, B, C, C Lighter and D) and competitor spread (E, F Butter, F Salted, F Unsalted, F Light, G and G Light) % salt in moisture.

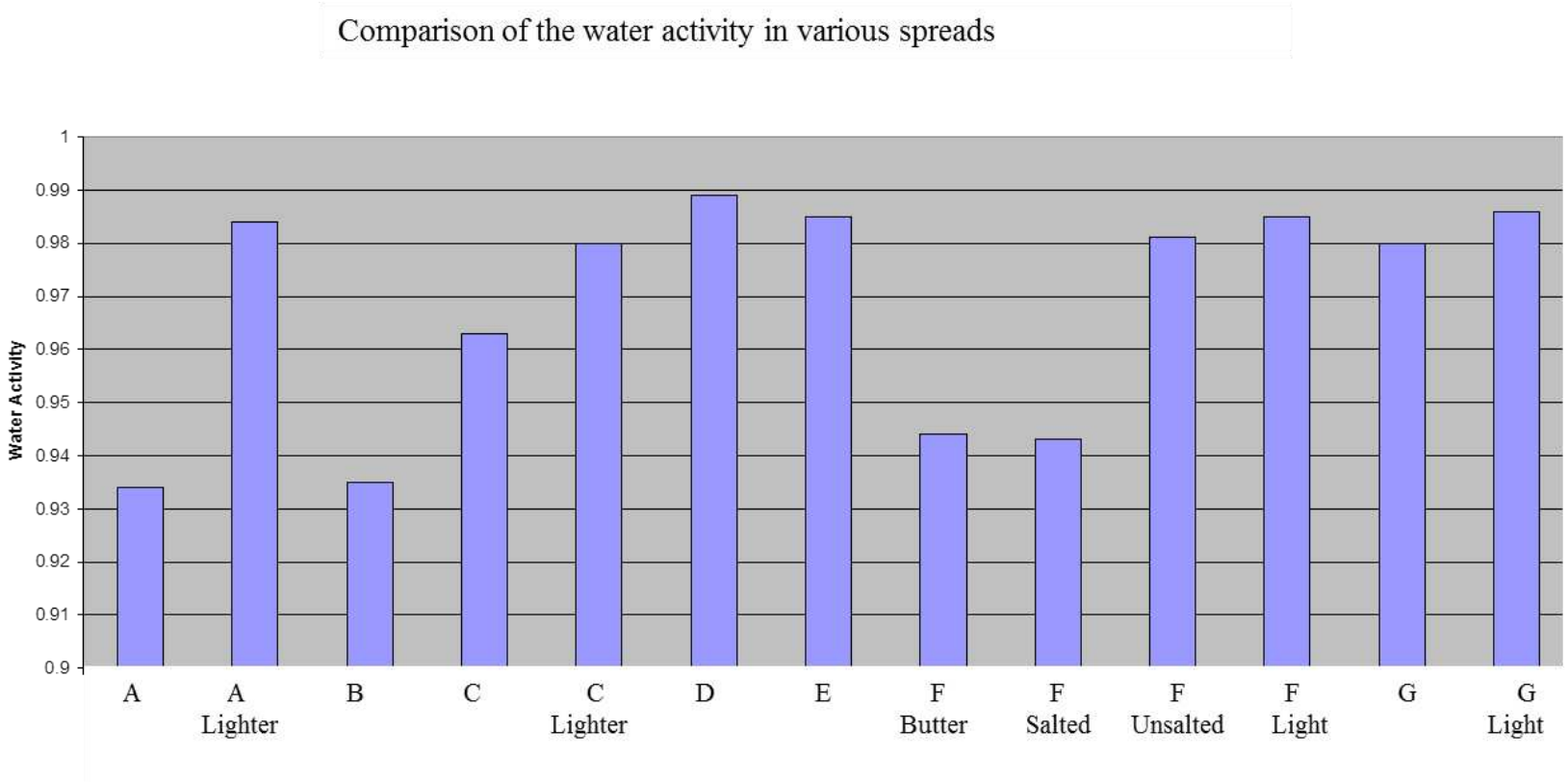


Figure 5.6 Comparison of standard spread formulation (A, A Lighter, B, C, C Lighter and D) and competitor spread (E, F Butter, F Salted, F Unsalted, F Light, G and G Light) water activity.

5.4 THE PROCESS CHANGE

5.4.1 A traditional spread processing via pasteurisation

Cream is a bi-product of milk processing plant and one of the main ingredients in the processing of spread (Henning and Dave 2006). Prior to processing, the cream is pre-pasteurised at 83ºC (or lower) for 20 seconds and then stored in an aging tank for a maximum of 48 hours. The cream is then mixed with water, buttermilk, emulsifier powder, oil and vitamins and pasteurised at 83ºC. The separated buttermilk, a bi-product of the production process, is further recycled and pasteurised at 72ºC for use as an ingredient in the spread processing (Figure 1.5).

5.4.2 A new technology: scraped surface heat exchangers for spread processing

The scraped surface heat exchangers (SSHE) consist of a jacketed cylinder with a rotating dasher holding rows of scraper blades. The product is pumped through the cylinder while the heating or cooling medium is circulated between the cylinder and the jacket. The medium can be steam, water or a refrigerant such as ammonia or Freonn etc. (Harrod 1986). The SSHE crystallizes the fat at the working station by mixing under a rapid cooling system with liquid nitrogen; this rapid cooling produces a lower and finer droplet size (Figure 1.6 and 1.7).

After the quality taint of ‘cheese flavour’ spread, the spread making for Spread A and A lighter was changed to SSHE to achieve a finer droplet size distribution to a minimum of 90% $\leq$ 5 µm within the product to limit the available water for

microbial growth. Spread C and C lighter were already produced by the SSHE method and hence had better droplet distribution (Figure 5.3).

5.4.3 Change in the product droplet size

Droplet size was determined as in Section 3.2.1.6 as an ongoing every day monitoring parameter after the process was swapped over for a trial. It was noted that 95% of the droplet size for a 24-hr run batch had a droplet size less than 10 µm (Figure 5.7), in an average nearly of 90% samples over a 11 weeks shelf life period.

During process intervention or batch change over the target level dropped as low as 80% ensuring a continuous process is vital to achieve product of good quality. The droplet size was one of the key tests undertaken daily for a quality and safety monitoring of the product.

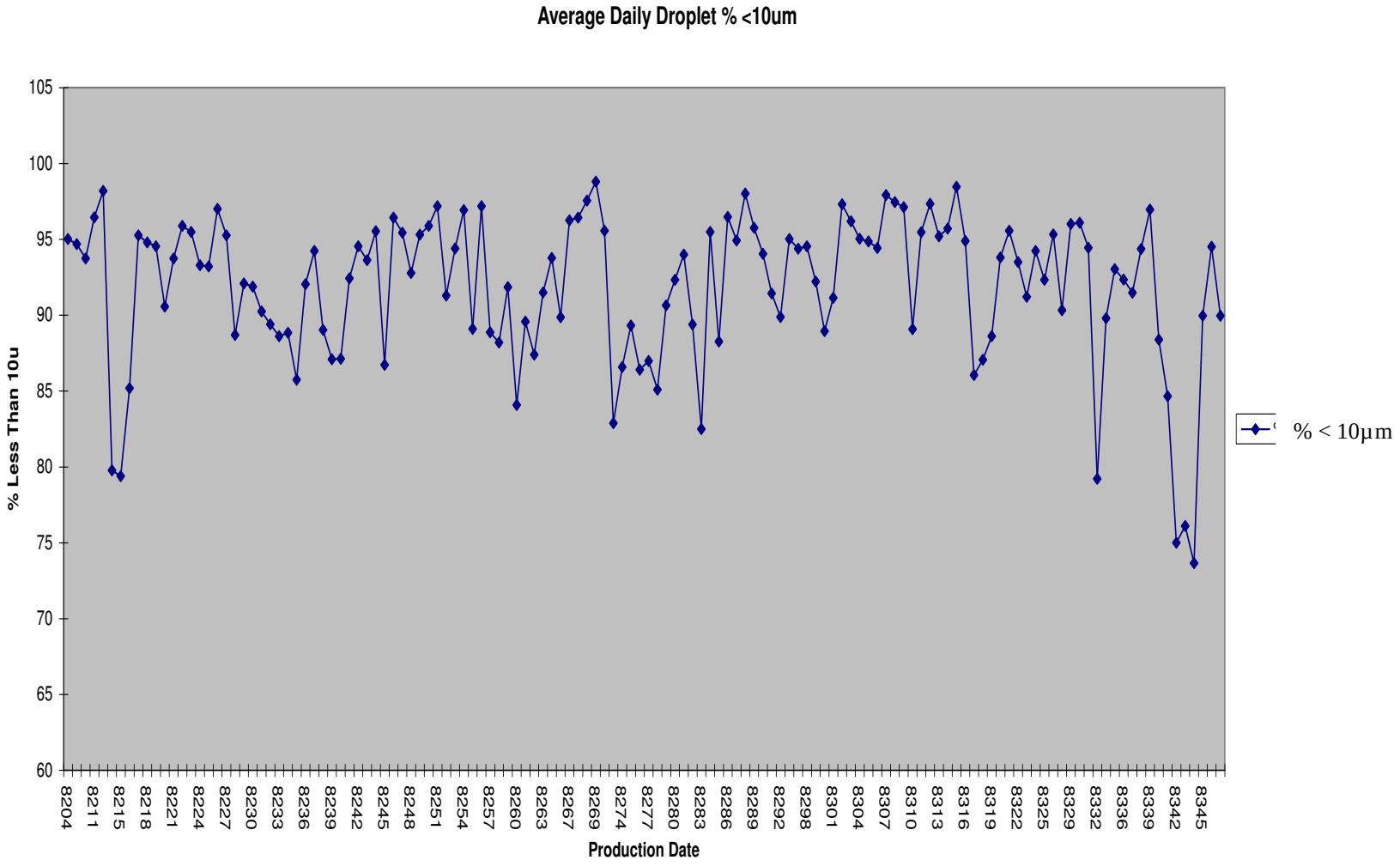


Figure 5.7 Droplet size result summary from SSHE spread processing.

5.4.4 Methyl ketone generation

Table 5.6 show methyl ketone (MK) generation in the traditional (STD) and new process formulation of product. It was observed that the STD product produced more MK compared to the new product produced under the new processing system. Milk was used as a control in this experiment. The two methyl ketone responsible for the distinctive ‘cheese flavour’ smell were pentanone and hexanone.

The new finished products were further analysed for methyl ketone generation (Figure 5.8). This showed roughly a 50% reduction in the generation of the ‘cheese flavour’’ methyl ketones, i.e., pentanone and hexanone were observed in 100 new and STD products. On day 7 no MK generation was found for the new formulation product and the control, whereas the standard product showed the presence of breakdown of the product. The same was also observed on the 14-day test. Hence the new products were more robust against the growth of the causative organisms giving a ‘cheese flavour’’ spoilage aroma.

Table 5.6 Methyl Ketone (MK) generation in the traditional (STD) and new process formulation of product.

Product Type	MK Generation	Average Methyl Ketone (MK) Results (mg/kg)							
		MEK	2-Pentanone	MK	2-Hexanone	2-Heptanone	2-Nanonone	2-Undecanone	2-Tridecanone
STD	7 days	0	0.024	0	0	0	0	0	0
NEW		0	0	0	0	0	0	0	0
STD	14 days	0	0.044	0.024	0.043	0	0	0	0
NEW		0	0.021	0.038	0.019	0	0	0	0

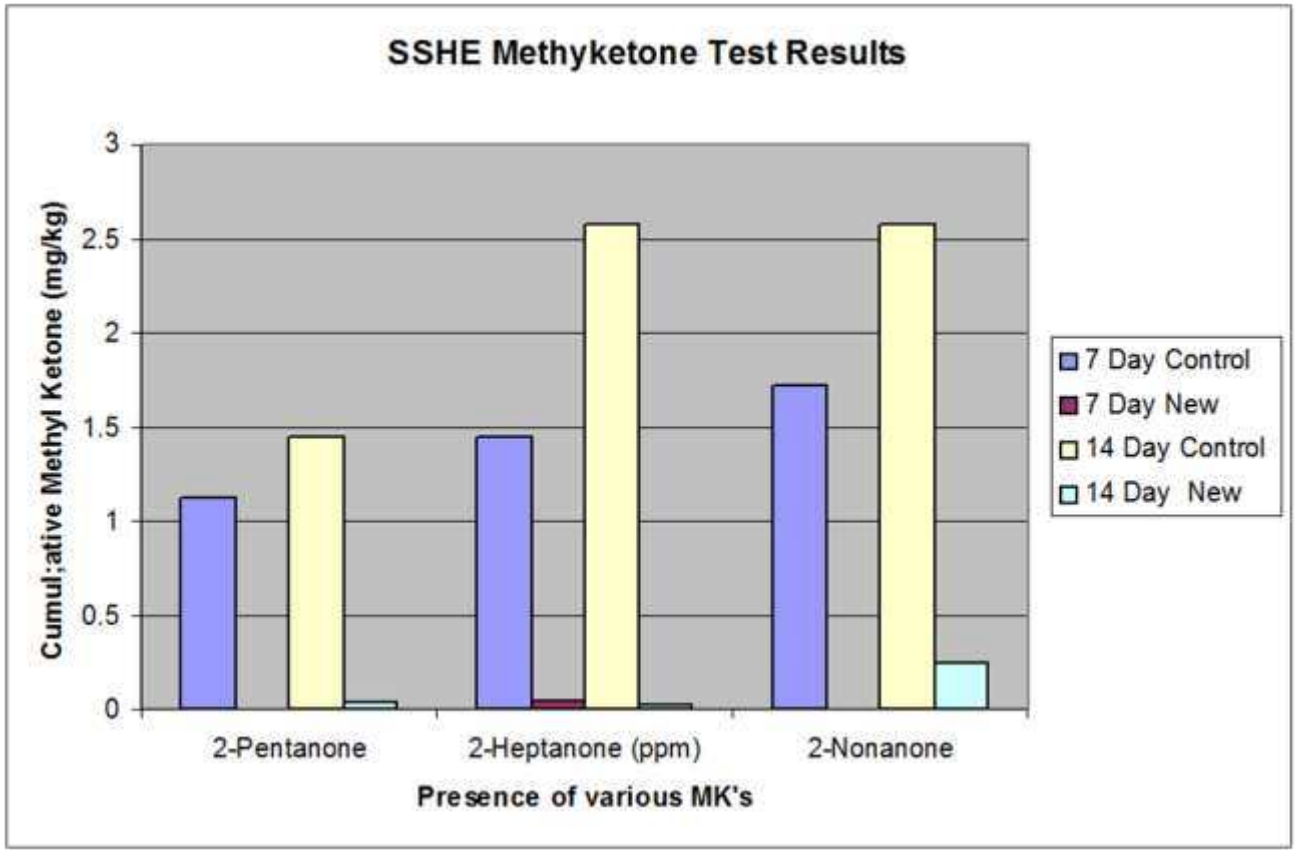


Figure 5.8 Methyl ketone generation for trial product

5.5 DISCUSSION

Rheological properties are the resistance to motion caused by internal friction between molecules as they pass each other (Prentice 1984). It is a measure of the internal drag, the action of emulsifiers and gelling agents and also the physical state of the product's constituents. Many factors affect the rheological properties of dairy products e.g. temperature, age, solids content, fat globule size, ratio of liquid to molten fat at a given temperature, gel formation and processing conditions (McSweeny and Fox 2009). Emulsion breakdown is also influenced by environment as pH, temperature and processing conditions (Rousseau 1999). They lower the interfacial tension between the oil and water phases and also form a mechanically cohesive droplets thus preventing coalescence.

Spreads can become rancid by undergoing breakdown of the fat liberating free fatty acids and peroxides that change both physical and organoleptic properties of the product. The FFA's are further broken down into smaller volatile molecules such as aldehydes and (methyl) ketones (Stead 1986; Allen and Hamilton 1994; Brocklehurst 1995) giving rise to unpleasant taints. Methyl ketones gives the distinctive 'cheese flavour' that can be detected at low levels organoleptically (Table 5.1) (Molimard and Spinnler 1996; Engel et al. 1997).

One of the routes of product breakdown is contamination by microbiological organisms (Aprigny and Jaeger 1999). Certain groups of microorganisms can be characterized by the way they metabolize the product fat and break it down (Allen

and Hamilton 1994; McKenna 2003). The measurement of specific breakdown products such as methyl ketones can be a useful tool in predicting the nature of the microbiological contamination (Allen and Hamilton 1994). These methyl ketones are generated by the oxidation of free fatty acids that in turn are released by the degradation of the fat phase of the spread. The carbon chain compounds responsible for a distinctive fruity aroma are C6, C8, C10 and C12. Among them C8 and C10 give a 'cheese flavour' cheese smell (Patton 1950). Free fatty acids (FFA) derived from milk fat generally are believed to contribute to the flavour of most cheeses such as Stilton (Engel et al. 1997).

The key lipolytic organisms associated with dairy product spoilage are Pseudomonas spp, Micrococcus spp, Bacillus spp, moulds such as Penicillium and Aspergillus spp and yeasts such as Candida and Saccharomyces spp. All these organisms except Bacillus spp are killed by pasteurisation, as Bacillus spp is a spore former (Allen and Hamilton 1994; McKenna 2003).

In good microbiologically clean milk, the lipolysis may also be due to the intrinsic milk lipoprotein lipase. High FFA levels can arise either from high levels in the milk, pre-manufacture lipolysis or post pasteurisation lipolysis (Allen and Hamilton 1994). The latter mainly is caused by microbial contamination during or after processing or by contamination of heat resistant enzymes from psychrotrophic bacteria that are able to grow in storage silos below 5ºC (Chavez 1994). Therefore the problem may often be intermittent and difficult to trace.

The standard products and ‘cheese flavour’ samples were taken over a month which also excluded batch variation for processing parameters such as people, shift and ingredients such as skimmed milk, sweet cream, sweet cream buttermilk, cream, buttermilk, oil, water. It was noted that most of the ‘‘cheese flavour’’ were developed when the product was 30 days old. But there were incidences of strict ‘cheese flavour’ smell in samples as young as seven days old. However, the off quality taint of ‘cheese flavour’ were greater (78%) with 1 kg tubs compared to a 500g tub, but this was not a regular incidence as the quality rejection on ‘cheese flavour’ varied through the months and time of production. This may indicate that this is a surface phenomenon which may be due to the spoilage that is accelerated by a combination of shearing and air i.e. oxygen and light exposure and environmental contamination. The ‘cheese flavour’ sample aroma varied through various months of the year along with different production time and hours, indicating spoilage being accelerated by a combination of shearing and air as oxygen and light exposure and environmental contamination. The main causative organisms isolated in Section 4 by screening the finished products, ingredients, personnel, air, water and packaging were Bacillus spp (vegetative state), Staphylococcus spp, yeasts and moulds that are mainly aerobic microorganisms. Hence it is highly likely that due to growth of these organisms in the oxygenated layer causing the product breakdown and changing the structure of the food matrix.

The top layer of the spread was significantly softer (10%) in the 'cheese flavour' products to that of the standards compared to the middle and bottom layer within the tub with the variability standard deviation being ~ 14% (Figure 5.1).

The isolates (Staphylococcus equorum, Staphylococcus pasteuri, Staphylococcus sciuri, Bacillus subtilis and Bacillus licheniformis. Pichia guilliermondii, Candida guilliermondii, Candida parapsilosis and Candida ortho parapsilosis) showed significant levels of lipolytic activities (Table 4.2). Lipase enzyme hydrolyses glycerides to form free fatty acids; in butter and other dairy products this can cause rancidity (Charteris 1996). The characteristic odours of rancid dairy products are caused by the release of butyric acid - a volatile fatty acid. These organisms were further used to determine the robustness of the product structure (Figure 5.9).

As milk fat constitutes only 1% of the fat phase makeup and milk fat contains circa 3.5% C12 compared with 17.4% in the oil blend it was further postulated that the hard oil blend was the most likely source for the generation of methyl ketones (Table 5.4). This contrasts with findings in Section 4.4.1. as in the ingredient testing oil came out as low risk having had very low microbial contamination, with only 10% of samples positive with Bacillus spp and all the counts were less than 10^3 cfu /g as required by the product specification. Therefore it was thought that oil as an ingredient is not high risk, but when blended in the product with other dairy formulations if there are any of the causative agents present they can breakdown the product and result off flavour'.

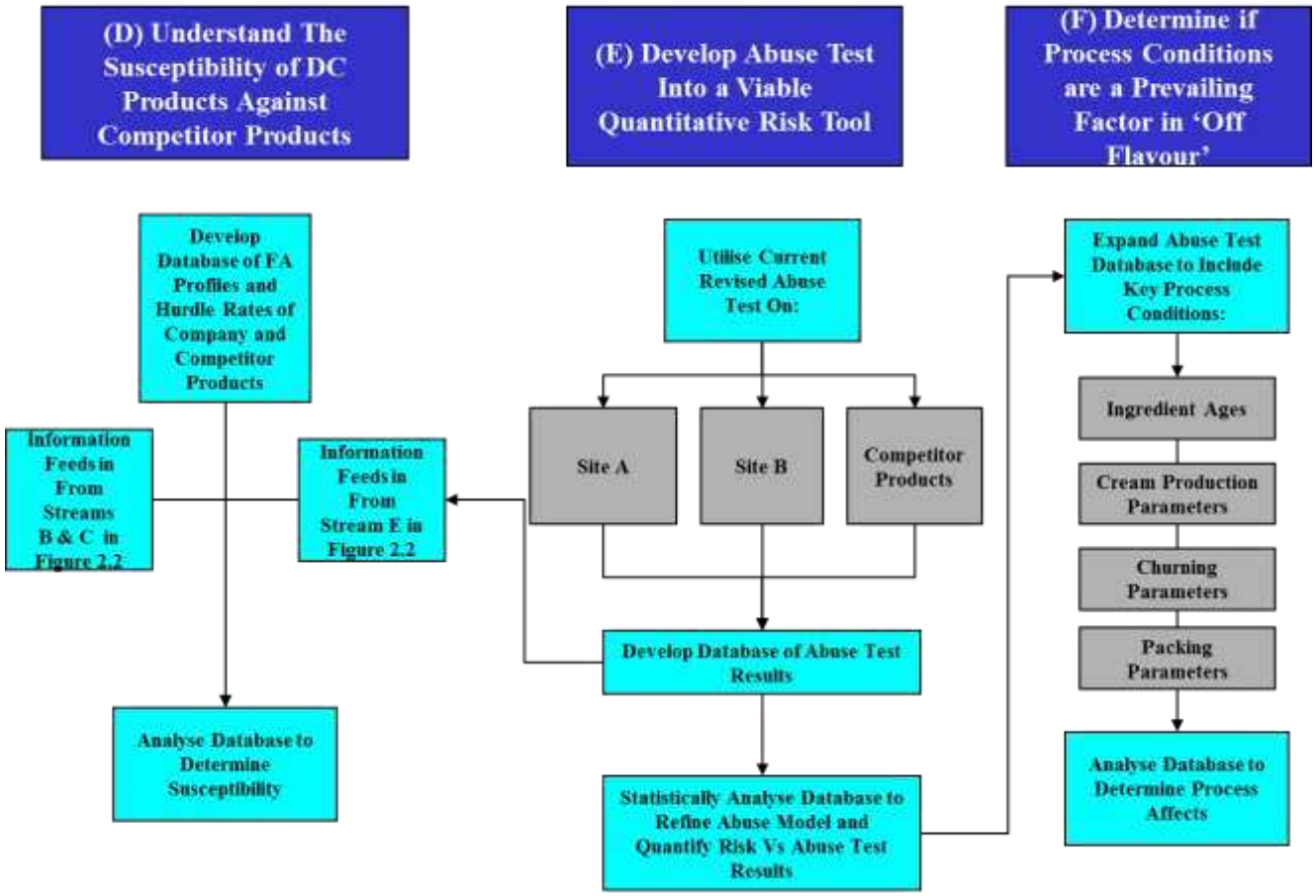


Figure 5.9 Study Layout for the chemistry analysis to determine the product hurdle / robustness.

Spread is a ‘water-in-oil’ product, the water being present as a well-dispersed fine droplets throughout the fat phase. The inability of microorganisms to move between droplets is a major intrinsic preservation factor (ICMSF 2005). Fat can act as a barrier to microbial growth, resulting in much more stable fat continuous systems than water continuous systems (Rajah 2002). This generally limits microbial growth, but does not necessarily exclude growth under extreme conditions (ICMSF 2005). The ability of a microorganism to grow in an emulsion depends partly on the volume of the water droplet it finds itself in. Microorganisms cannot grow in emulsions with a droplet size of less than approximately 10μm (Brocklehurst 1995, McSweeney 2009). Water droplets are larger in products containing less fat, thus providing cells with increased space and more water-soluble nutrients from the ingredients. Temperature, redox potential and pH influence the energy-dependent growth of microorganisms in ‘water in oil’ emulsions (Charteris 1995, Rousseau 1999).

Emulsifiers are surface active compounds used to reduce the interfacial tension between the water and fat phase. It stabilises the liquid emulsion prior to crystallisation for a homogenous product and to achieve finely dispersed water droplets in order to improve the microbial keeping properties of spread (McClements 1999). The preservation system depends on the presence of a fat-continuous matrix and any water present is in the form of very finely dispersed droplets. Due to this dispersion, contaminating microorganisms are restricted in growth either by space limitations or by exhaustion of the nutrients in the droplets.

shells of fat crystals covering water droplets prevent these from merging together (Juriaanse and Heertje 1988).

Spread ‘A’ and ‘B’ had a poorer droplet size distribution 95% ≤ 10 μ (Figure 5.3) with former being significantly lower than the other market competitors around 68%, whereas ‘C’ and ‘C lighter’ achieved 98%≤5 μ level.
This difference in droplet distribution may be linked to the process technology utilised to produce the product. The SSHE process gives finer droplet size distribution and leads to a greater degree of microbial stability and hence more robustness against ‘cheese flavour’’ causative organisms.

Spread A and A lighter after being produced by the scrape surface heat exchanger instead of the traditional pasteurisation churn process gave , a finer droplet size distribution to a minimum of 90% ≤ 5 μ and showed a 50% reduction in the generation of the ‘cheese flavour’’ methyl ketones, i.e., pentanone and hexanone. Hence the new products were more robust against the growth of the causative organisms giving a ‘cheese flavour’’ spoilage aroma.

Based on the findings in Chapter 4 and 5, the study will further focus into challenge testing the current product with the identified contaminants in Chapter 4 produced under the traditional processing and SSHE processing and evaluate the spoilage rate and methyl ketone generation.

CHAPTER 6 CHEMICAL CHALLENGE TEST

The causative organisms Staphylococcus equorum, Staphylococcus pasteuri, Staphylococcus sciuri, Bacillus subtilis and Bacillus licheniformis, Pichia guilliermondii, Candida guilliermondii, Candida parapsilosis and Candida ortho parapsilosis were further used as a reference strain for microbial challenge testing to determine the product robustness produced by SSHE process. Methyl ketone measurement was used as a determining tool in this section.

All the isolated and identified bacteria were grown on Nutrient Agar as a pure culture overnight at 30ºC. The cultures were washed with 1 ml of sterile MRD and transferred into 250ml of Nutrient Broth and incubated again at 30ºC overnight in a New Brunswick floor standing orbital shaker at 200rpm as per method 3.3.1. The strains were inoculated into 10 g of spread or oil or other ingredients prepared as per method 3.3.1 with the organisms identified in Sections 4.2 and 4.3. All samples were then stored and tested for methyl ketone (MK) generation (method 3.2.1.2).

6.1.1 Ingredient challenge test

Dairy (cream, buttermilk, skim milk) and non-dairy (oil) ingredients were challenge tested using isolated microorganisms and the effect of temperature abuse accelerating the process was also further examined.

6.1.1.1 Microbial challenge test

All the ingredients (skimmed milk, cream, buttermilk and oil) were challenge tested with the single purified isolated strains as per Section 3.3.1 and incubated at 37ºC for 7 days prior to analysing for MK as per Method 3.3.4. The time and temperature combination was used as an accelerated spoilage conditions for the organisms that is set by the company accelerated test method.. The results are attached in Appendix 10.6 and Table 10.3; each test per product per organism was replicated five times. From the Table it was observed that not a significant amount of FFA was detected after 7 days of incubation. Skimmed milk, cream, buttermilk and oils showed no sign of the presence of MK that generated spoilage.

6.1.1.2 Temperature abuse test

The cream, buttermilk and skimmed milk were abused by storing at 4 different temperatures as in Section 6.1.4 at 5ºC, 10ºC, 20ºC and -18ºC and tested for pH as per Method 3.2.1.3 and FFA as per Method 3.2.1.2. For the trial the ingredient's age since its last pasteurisation was a minimum of 3 days to replicate the usage under current spread processing. An additional temperature of 10ºC was used in this process which denotes the storage silo temperature. No significant change was noted at 5ºC and -18ºC and at 20ºC the cream separated at day 1 and no further testing was performed (data not presented). While storing under 10ºC it was observed that a significant pH drop was noTable from an initial pH 7 to as low as 4.5 in cream in day 5, whereas the pH was still near to neutral at 6.1 for buttermilk and skimmed milk (Figure 6.1). FFA generation in skimmed milk and

buttermilk was not noticeable, but cream generated significant levels of FFA where these went as high as 0.45% from 0.2% in 5 days. From the experiment in the generation of MK under various spread processing parameters (Table 5.5) it was noted that FFA levels above 0.04 mg/kg showed noticeable spoilage detectable via sensory tests.

6.1.1.3 Oil blend challenge test

The oil blend and butter oil were challenge-tested with all the isolated causative agents. Dairy samples (skimmed milk and cream) were used as a control to demonstrate that the microbes were not breaking down solely the dairy product to cause spoilage as per method 3.3.4. From Figure 6.2 it was clearly observed that the dairy products in total (skimmed milk and cream) showed no generation of methyl ketones showing a similar trend to Table 10.3 in Appendix 10.6 when the ingredient challenge test was performed. But both the butterfat and oil blend were utilised as a source of nutrients for the spoilage and general microorganisms. The levels of the C12 and C14 based MKs is reflective of the initial concentration of C12 and C14 in the base oil i.e. butter fat has a lower level of C12 than hard oil, so generates less 2-undecanone but has higher levels of C14 so generates more 2-tridecanone than hard oil. The high levels of 2-tridecanone compared with the low levels of C6 to C10 and the fact that 2-tridecanone is found in lower quantities in the ‘cheese flavour’ samples would suggest that as the metabolic pathways progress the MKs are further reduced to shorter chain MKs. This demonstrates that the generation of MKs is linked to the fatty acid profile of the oils.

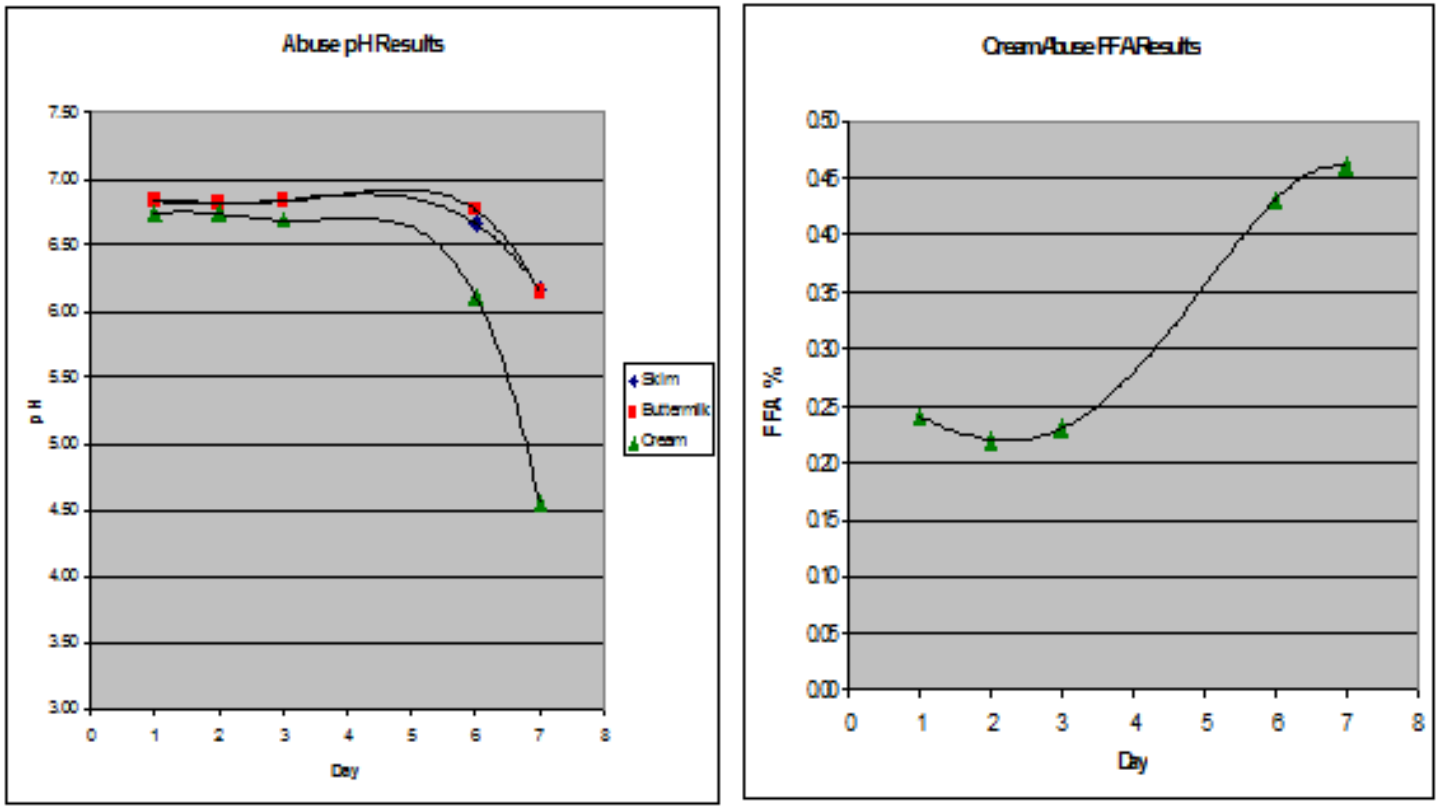


Figure 6.1 FFA and pH development in cream under storage at 10ºC

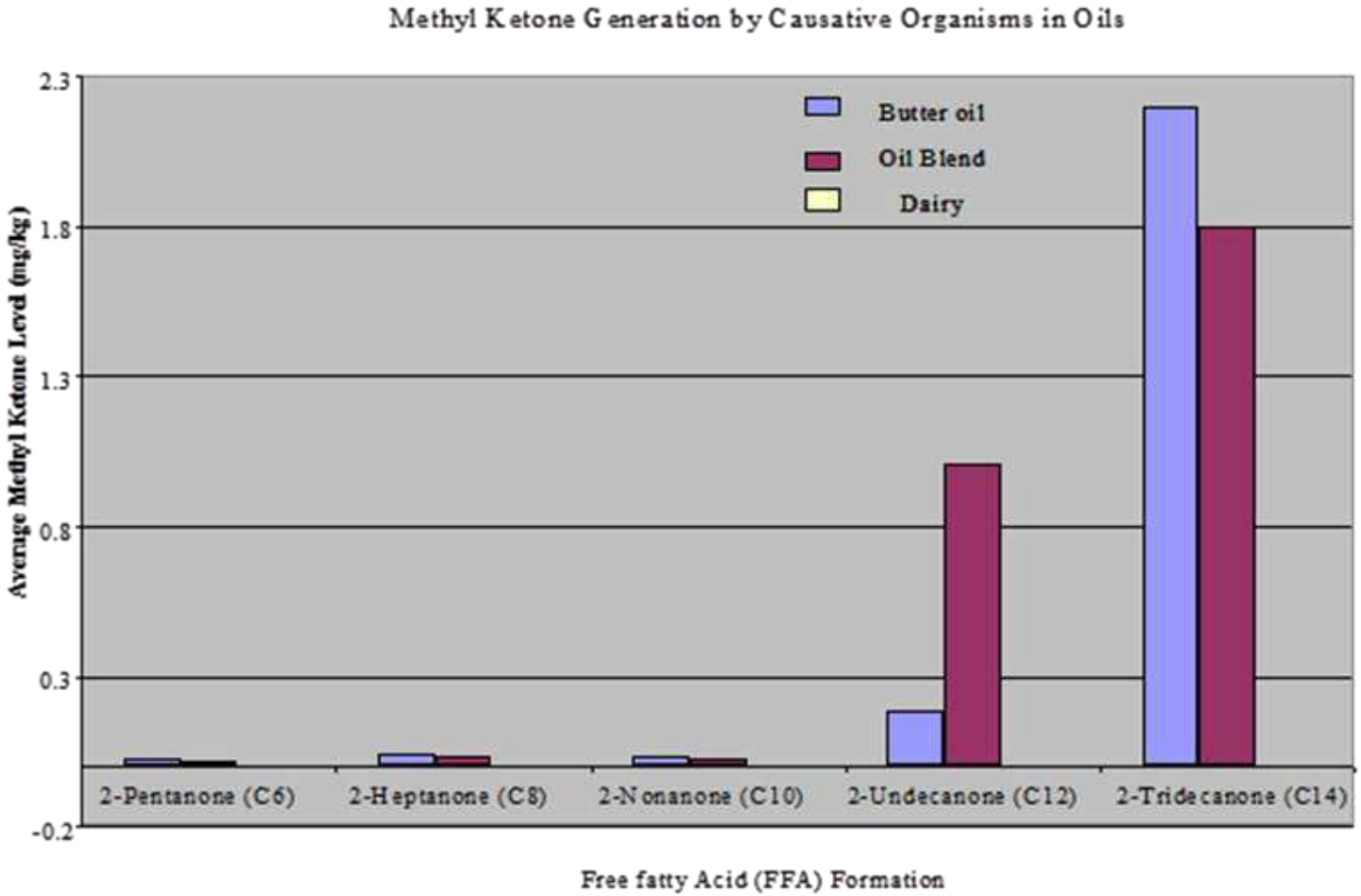


Figure 6.2 Analysis of methyl ketone formation in oil, buttermilk and cream inoculated with the causative spoilage microorganisms

6.1.2 Spread challenge test

Challenge tests for spreads were done in two stages: as single isolates and also as composite microbial samples. For the test a total of 17 spreads (A, A Lighter, B, C, C Lighter, D, H, I, I omega 3, E, F Butter, F Salted, F Unsalted, F Light, G and G Light and J Table 5.2) were used that included the suspect spread samples along with the market competitors and some lighter salt preparations as well. Spreads were sub-sampled into a sterile container (100g) to avoid contamination. 10g of the sample was prepared according to Section 3.1.1 and plated out on NA and selective agars for various microorganisms to perform a total count prior to inoculation with the challenge microorganisms. The spread samples used for the 'cheese flavour' abuse and challenge tests as per Section 3.3.4.

For microbial cocktail challenge testing all the organisms Bacillus spp, Staphylococcus spp, yeasts and moulds were used as a cocktail as per Method 3.3.2 to mimic the natural contamination from the environment.

A total of two challenge test was performed, firstly looking at the comparison of the development of MK over a shelf life of 10 weeks on various spreads by the causatives organisms and lastly the effect on the speed of MK generation under temperature abuse along with sensory differentiation.

6.1.2.1 Microbial challenge test

A replicate of 5 samples each spreads (A, A Lighter, B, C, C Lighter, D produced by UK; H, I, I omega 3 are produced by EU and competitor product E, F, G and J) (Table 5.2) were tested over 10 week after being inoculated by the cocktail of microorganisms as per Section 3.3.2 and 3.3.4. Table 6.1 summarises the average of the 5 replicates performed in sensory and MK generation test.

The distinctive 'cheese flavour' was noted at the methyl ketone level ≥ 0.08 mg/kg which is in line with the findings in Section 5 (≥ 0.04 mg/kg). In most cases the level of Mk increased from 0.01 to 0.08 within a week as the tests were analysed weekly.

It was observed that Spread A, and C Lighter started to show the presence of MK along with typical 'cheese flavour' odour from week 2. On the other hand spread A Lighter developed the presence of potential spoilage at week 3, spread B at week 8 and spread 'J', the only competitor product, at week 6. No other product has shown any tendency to develop 'cheese flavour'.

These results show that formulation in the spread A and C lighter are more prone to microbial spoilage than the other products available making them more vulnerable to contamination issues. To further evaluate the effect of each single microorganism in the product, single isolated purified colonies as per 3.3.1 were used to inoculate the same spreads as above and the rate of 'cheese flavour' development and MK generation was observed.

The results in Table 6.2 showed similar pattern to the results in 6.1 on the days the off flavour and significant MK generation was observed on the higher risk products as A, A Lighter, C Lighter and J.

No noticeable change was observed on the spreads D, H, I, I omega 3, E, F and G (data not presented). Staphylococcus spp mainly related to the factory and personnel hygiene related showed the development of cheese flavour within spread A, A lighter, B and D within 7 days in sensory testing with the level of MK (mg/kg) 0.010 to 0.012. Similar pattern and level was also observed with the mould challenge test samples. Spread J also developed significant levels of cheese flavour with mould contamination.

On the other hand Bacillus spp and yeast generated significant cheese flavour and MK above the level of 0.08 mg/ kg as per Table 6.1 in the spreads A, A lighter, B, C, C lighter, D, H and J within 21 days..

6.1.2.2 Temperature abuse test methylketone generation

For the next set of challenge testing spread A, A Lighter, B, D produced by UK market and E, F and G competitor products (Table 5.2) were taken and inoculated with the cocktail of microorganisms and the levels of different methyl ketones were analysed. The samples were ‘cheese flavour’ abuse tested as per Method 3.3.4.

Products were incubated throughout the product's life at room temperature 20ºC, refrigeration temperature 5ºC and at –18ºC. No significant changes to the products were observed at temperatures of 5ºC and at –18ºC (results not presented as the MK generation was static all the way through the shelf life). On the other hand, at room temperature 20ºC, (Figure 6.3) in three spreads (A, A lighter, B) there was higher generation of C8 to C12 MKs.

The common factors to all three of these products are the hard oil blend and the addition of butterfat. The fact that the rest of the samples which contained high proportions of butterfat have not generated the high level of 'cheese flavour'' MKs would substantiate the hypothesis that the hard oil blend is the main source of the MKs generation.

6.1.2.3 Temperature abuse sensory test

A temperature abuse sensory test was performed in 20 samples of spread type A, A lighter, B, C lighter and a competitor product J. the level of MK generation was observed at 3 different temperatures, refrigeration (5ºC) temperature, room temperature (20ºC) and -18ºC freezing temperature to mimic general consumer abuse as the product is suitable for freezing. The results are summarised in Table 6.3.

At 20ºC the generation of product spoilage was indicated with an increased level of MK generation (method 3.2.1.2) and an early indication of 'cheese flavour' potential can be assessed. 56% of spread 'A' showed 'cheese flavour' within 15 to

36 days, similar to the level on MK generation in Table 6.1where the level of 0.08 mg/ kg was detected in week 2. In other standard products such as ‘A lighter’, 25% showed signs of a fruity smell around 26 days and 25% of product spread ‘B’ at 54 days. Only one other competitor product from the market, product J (20%), showed signs of spoilage at 44 days. Twenty percent of ‘C Lighter’ samples showed signs of being off at 26 days. No other product showed any tendency to turn ‘cheese flavour’.

The samples were also stored at refrigeration temperature throughout their life. The off-smell still developed in spreads A, A lighter and B but at a much slower rate. Only 15% smelled of ‘cheese flavour’ cheese at 52 days. All other products were satisfactory, i.e., no smell was observed. On the other hand only 1% of A’s frozen samples developed the distinct off smell.

6.1.2.4 Temperature sensory abuse test under aerobic / anaerobic conditions

O one hundred spread ‘A’ samples were further analysed by performing the product ‘cheese flavour’ abuse test as per Method 3.3.2, where the sample product was tested at 3 different temperatures: 20ºC (normal room temperature as per ISO17025), 5ºC (Industry standard chill chain) and -18ºC (freezing temperature) and also spread samples without a cover leaf were examined to understand the rate of spoilage at various temperatures and conditions.

Table 6.4 summarises the results of sensory ‘cheese flavour’ taint test for product abuse testing. It was noted that when the samples were incubated at an elevated temperature (20ºC) the rate of product breakdown and formation of bi-product FFA that generates the distinct ‘cheese flavour’ accelerated compared to the refrigeration and freezing conditions.

An intact spread tub of spread A showed the presence of spoilage at Week 3 when stored at 20ºC since the first date of production compared to only 3 days when the spread was aerated with the spatula and the coverleaf taken off and 10 days with the coverleaf on. On the other hand under refrigeration temperatures at 5ºC no spoilage was noted throughout the 10 week shelf life of the un-abused, unopened tube. But 3.5% of the samples developed a distinguishable ‘cheese flavour’ taint at week 8 in the abused, no coverleaf sample compared to only faint development in 2% samples at day 70. No change of the product was noted under freezing conditions.

The un-abused frozen spread samples were stored at 20ºC after the end of 10 week shelf life and nearly 60% of them showed distinguishable ‘cheese flavour’ taint after 3 weeks compared to 1 week for the abused samples.

6.1.2.5 Effect of spread layers in a tub

A single tub of spread was initially divided into three Sections (top, middle and bottom) and each Section was cut into three equal Sections. Around 50 samples were analysed of which 25 were ‘cheese flavour’ samples and 25 standards. They were analysed for the presence of the causative microbes and enumerated. Methyl ketone analysis was also performed. The difference in the level of the microbes and the formation of the methyl ketone throughout the sample was not significantly different between the different Sections (data not presented).

Table 6.1 Comparison of MK generation in various products over a 10 week period with cocktail organisms

Products	Days tested for Odour and MK (mg/kg)																			
	WK 1		WK 2		WK 3		WK 4		WK 5		WK 6		WK 7		WK 8		WK 9		WK 10	
	Odour	MK	Odour	MK	Odour	MK	Odour	MK	Odour	MK	Odour	MK	Odour	MK	Odour	MK	Odour	MK	Odour	MK
A	N/A	0.01	√	0.08	√	0.14	√	0.26	√	0.46	√	0.5	√	0.68	√	0.87	√	1.38	√	2.67
A Lighter	N/A	<0.01	N/A	0.04	√	0.08	√	0.12	√	0.19	√	0.24	√	0.28	√	0.36	√	0.45	√	0.86
B	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	√	0.08	√	0.12	√	0.26
D	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
H	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
C	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.02	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
C Lighter	N/A	0.02	√	0.08	√	0.12	√	0.18	√	0.22	√	0.24	√	0.38	√	0.42	√	0.49	√	0.53
I	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
I Omega 3	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
E	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
F Butter	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
F Salted	N/A	<0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
F Unsalted	N/A	<0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
F Light	N/A	<0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
G	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
G Light	N/A	<0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01	N/A	0.01
J	N/A	0.01	N/A	<0.01	N/A	0.02	N/A	0.02	N/A	0.02	√	0.008	√	0.15	√	0.23	√	0.28	√	0.3

Table 6.2 MK generation on variety of spreads from single organism challenge test

Spreads	*Staphylococcus* spp				*Bacillus* spp				Yeasts				Mould			
	1		2		B1		B2		Y1		Y2		M1		M2	
	Cheese flavour (Days)	MK (mg/kg)	Cheese flavour (Days)	MK (mg/kg)	Cheese flavour (Days)	MK (mg/kg)	Cheese flavour (Days)	MK (mg/kg)	Cheese flavour (Days)	MK (mg/kg)	Cheese flavour (Days)	MK (mg/kg)	Cheese flavour (Days)	MK (mg/kg)	Cheese flavour (Days)	MK (mg/kg)
A	7	0.11	7	0.11	21	0.09	21	0.08	21	0.07	21	0.11	7	0.11	7	0.12
A Lighter	7	0.10	7	0.11	21	0.08	21	0.09	21	0.08	21	0.11	7	0.10	7	0.11
B	7	0.10	7	0.10	21	0.10	21	0.07	21	0.09	21	0.11	7	0.12	7	0.12
D	7	0.12	7	0.10	21	0.11	21	0.07	21	0.08	21	0.10	7	0.12	7	0.10
H					21	0.08	21	0.07	21	0.07	21	0.11				
C					21	0.08	21	0.07	21	0.07	22	0.08				
C Lighter					21	0.08	21	0.08	21	0.08	21	0.09				
J					21	0.10	21	0.06	21	0.08	21	0.08	21	0.11	21	0.08

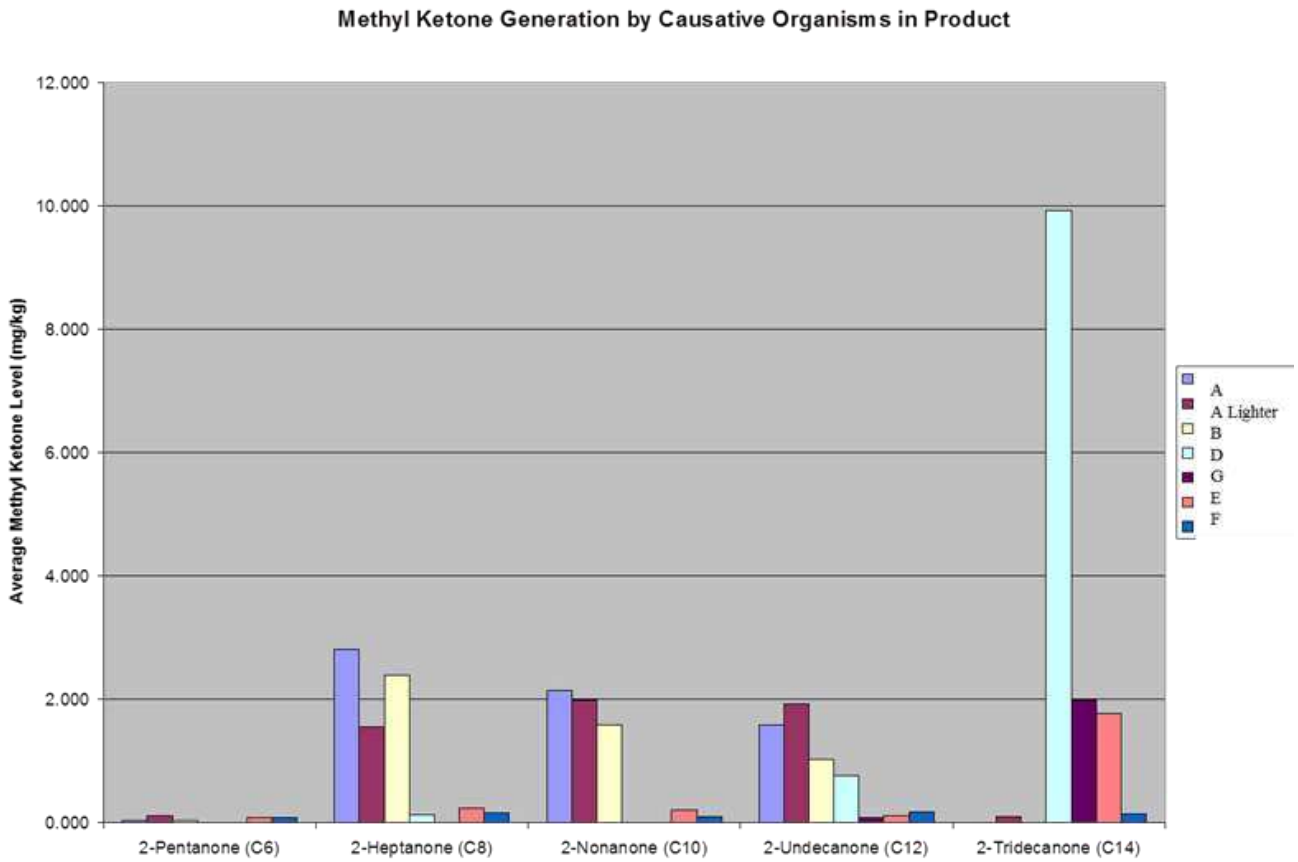


Figure 6.3 MK generation after challenge testing Standard spread A, A Lighter, B, D, and competitor products E, F and G with cocktail of causative organisms

Table 6.3 Cheese flavour development at various temperature

Spread	Taint Detected at various Temperature (°C)					
	20		5		-18	
	Days	% Positive	Days	% Positive	Days	% Positive
A	>15	56	40	25	>70	1
A Lighter	>26	25	> 52	15	N/A	N/A
B	>54	25			N/A	N/A
C	>54	15			N/A	N/A
C Lighter	>26	25			N/A	N/A
J	>44	20			N/A	N/A

Table 6.4 'Cheese flavour' taint formation observation in product abuse test

Abuse Test	Storage Conditions	Cheese Flavour	Samples (%)
Not performed	20°C	21 days	25
Performed & no cover leaf	20°C	3 days	55
Performed with coverleaf	20°C	10 days	55
Not performed	5°C	None	0
Performed & no cover leaf	5°C	56 days	3.5
Performed with coverleaf	5°C	70 days	2
Not performed	-18°C	None	0
Performed	-18°C	None	0

6.2 EFFECT OF ANTIMICROBIAL AGENTS ON THE CAUSATIVE MICROBES

Different antimicrobial agents were used to observe their detrimental effect on the growth of the microbes. Each strain was used separately in duplicate samples. Pure identified isolates from Sections 4.2 and 4.3 were used as an inoculum. Two different strains with 98% genetic similarity (as per Table 4.6 for bacteria and Table 4.7 for Y&M) were used as a duplicate rather than the same strain in order to understand the diversity within the group.

To prepare the initial inoculum, a pure colony of each bacterial strain was inoculated (spread plate) on Nutrient Agar and incubated for 24 hours at 37ºC. The overnight grown culture was washed with 10 ml of sterile MRD and transferred into 250 ml of sterile Nutrient Broth, incubated in a shaking incubator overnight at 37ºC and enumerated as per Method 3.3.3. For bacteria a culture concentration of 10^9 cfu/ml was taken to examine the antimicrobial agents. The initial inoculum was chosen to be very high to understand the bacterial death rate better and viable counting was achieved by performing a ten-fold dilution series to get counts between 30 – 300 cfu/ml in an agar plate.

For moulds, a 50 cfu/ml culture was taken, as it was difficult to count more than 50 large mould colonies on a plate. A total count of 10^7cfu/ml was achieved for yeast strains. For yeasts and moulds OGYEA and Chapex-Dox agar were used

respectively and cultures were prepared in the same way as the bacterial cultures mentioned above, but the incubation time was 5 days at 25ºC.

To test for antimicrobial effectiveness, Chapex-Dox, OGYEA and Nutrient Agar were prepared with 0.0, 0.01, 0.05, 0.1 and 0.2 % of potassium sorbate (total pH wasnot measured); 0, 1, 2, 5 and 10% sodium chloride , 0.55g/l of rosemary herb as per Section 3.3.3 and at different pH values such as 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0. The prepared agars were inoculated with 1ml of prepared inoculum of bacteria, yeast and mould strains (pour plate) and incubated at 30ºC for 24 hours for the bacteria and for yeast and moulds five days at 25ºC.

All the tests were carried out in duplicate and an average was taken for each cases

6.2.1 Effect of potassium sorbate

Most spreads available on the market contain potassium sorbate as an ingredient, as it is primary used as a food preservative (E number 202) (European Commission website). As per European Commission Regulation 1129 / 2011 amending Annex II of Regulation (EC) No 1333 / 2008 on food additives states maximum level of Potassium Sorbate (E 202) allowed in fat emulsions with fat exceeding 60% (excluding butter) is 1000 mg/l. Not only does it inhibit the growth of microorganisms for shelf life stability but it is also considered to be safe for consumption because of its long term safety record and non-toxic profile. The antimicrobial activity of sorbic acid and potassium sorbate is effective up to pH

6.5 but the antimicrobial activity decreases with lower pH (pH ≤ 4.5 for Listeria spp) (Charteris 1995).

Figure 6.4 illustrates the growth of bacteria and Y&M at various levels of potassium sorbate in the growth media. The total number of bacterial cells remained constant at all the levels showing no significance decrease (≤ 1 log cfu / ml). As an industry standard, a growth reduction ≥1 log is seen as significant (Campden BRI 2010 guideline).

The level of the total cell numbers for Y&M at various concentration also remain static.

6.2.2 Effect of sodium chloride

Salt is added in food as a natural preservative at a concentration of 1.5 to 1.8% of the whole product formulation. It helps to reduce the water activity of the product and hence acts as an antimicrobial agent. S. aureus was used as a control strain as this organism is known to have high salt tolerance.

Figure 6.5 summarises the growth pattern of vegetative cells of bacteria (S. aureus (control strain), S. equorum, S. pasteuri, B. subtilis and B. licheniformis), Y&M (Candida ortho parapsilosis, C. parapsilosis and P. guilliermondii).

The bacterial cells have shown a static level of total number between 0 to 2% salt concentrations in total. Whereas slight change in the total number was noted at 5% salt concentration but the total reduction was less than 1 log cfu/ml. The die off rate at 10% salt was variable with most of the Bacillus spp showing ≤ 2 log cfu / ml count reduction. The Staphylococcus spp mostly showed ≤ 2 log cfu / ml count reduction other than one strain of S. pasteuri where the growth remained constant. The yeast strains showed no change at 0 and 1% salt with a slight reduction (≤ 1 log cfu / ml) at 2 and 5% concentration other than one strain of C. parapsilosis and P. guilliermondii. The growth reduction at 10% salt was variable but all within the 1 log standard deviation error bar. No change in total number was noted for moulds within 0 to 5% salt with a total die off at 10% concentration. But due to the difference of the initial inoculmn levels of the moulds compared to the bacterial and yeast cells, these results are not directly comparable.

From this experiment it was noted that susceptibility of various strains at 10% NaCl varied significantly from strain to strain of the same species.

6.2.3 Effect of pH

Acidity or low pH has an antimicrobial effect. However the pH range which could be examined was limited to that of the food profile. The pH of the spreads could not go below 5 or above 6.5 due to formulation, stability and taste consistency that is favourable for the growth of most microbes. The details of the effect of variation pH on the growth of the causative microbes are described in this Section.

It was observed from Figure 6.6 that there was no reduction in the initial inoculum level of the microbes between the pH ranges of 5.0 – 6.5. There was little or no effect on the control bacterial strain S.aureus over the range of pH used (5.0, 5.5, 6.0 and 6.5). This showing that the isolated presumptive causative bacterial strains responsible for the 'cheese flavour' in spread can grow at a pH range of 5.0 to 6.5 and the pH of the product is 6.0 ± 0.5.

A similar growth pattern was observed for both yeast and moulds. They all showed increased survival at pH 6.0 and 6.5 and a negligible reduction in cell count at pH 5.5.

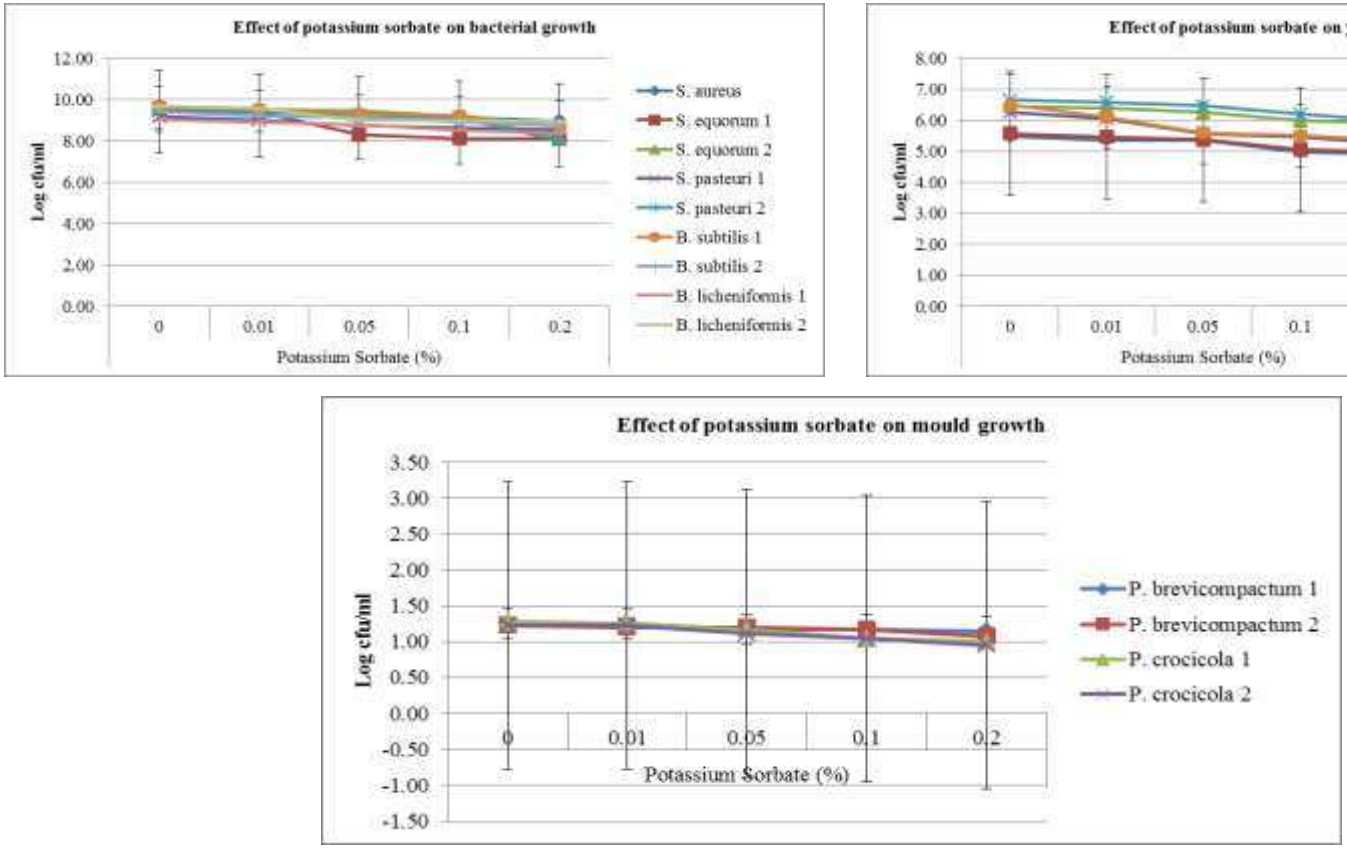


Figure 6.4 Effect of potassium sorbate at different concentrations on bacteria and Y&M growth.

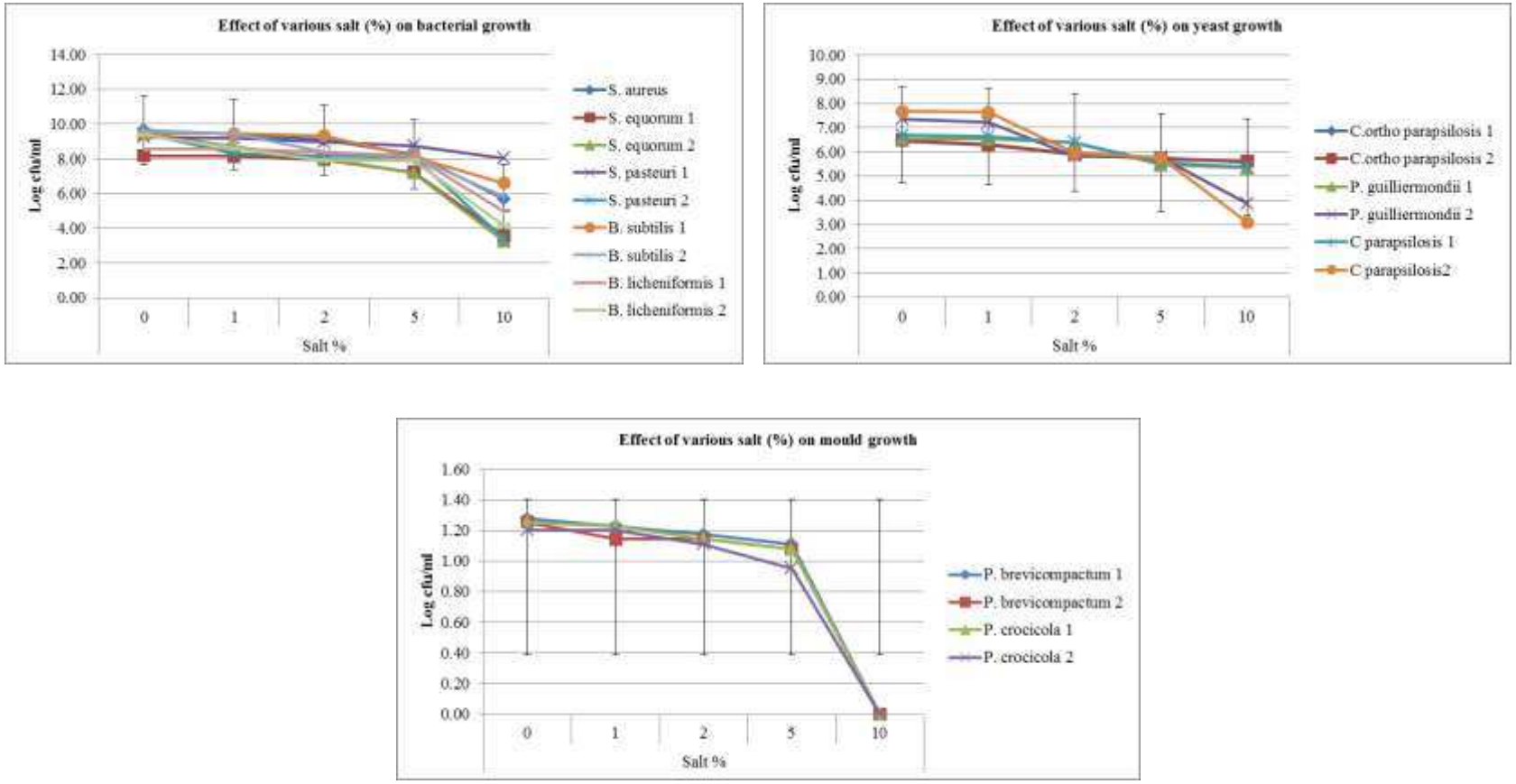


Figure 6.5 Effect of various salt concentration (%) on bacteria and Y&M growth

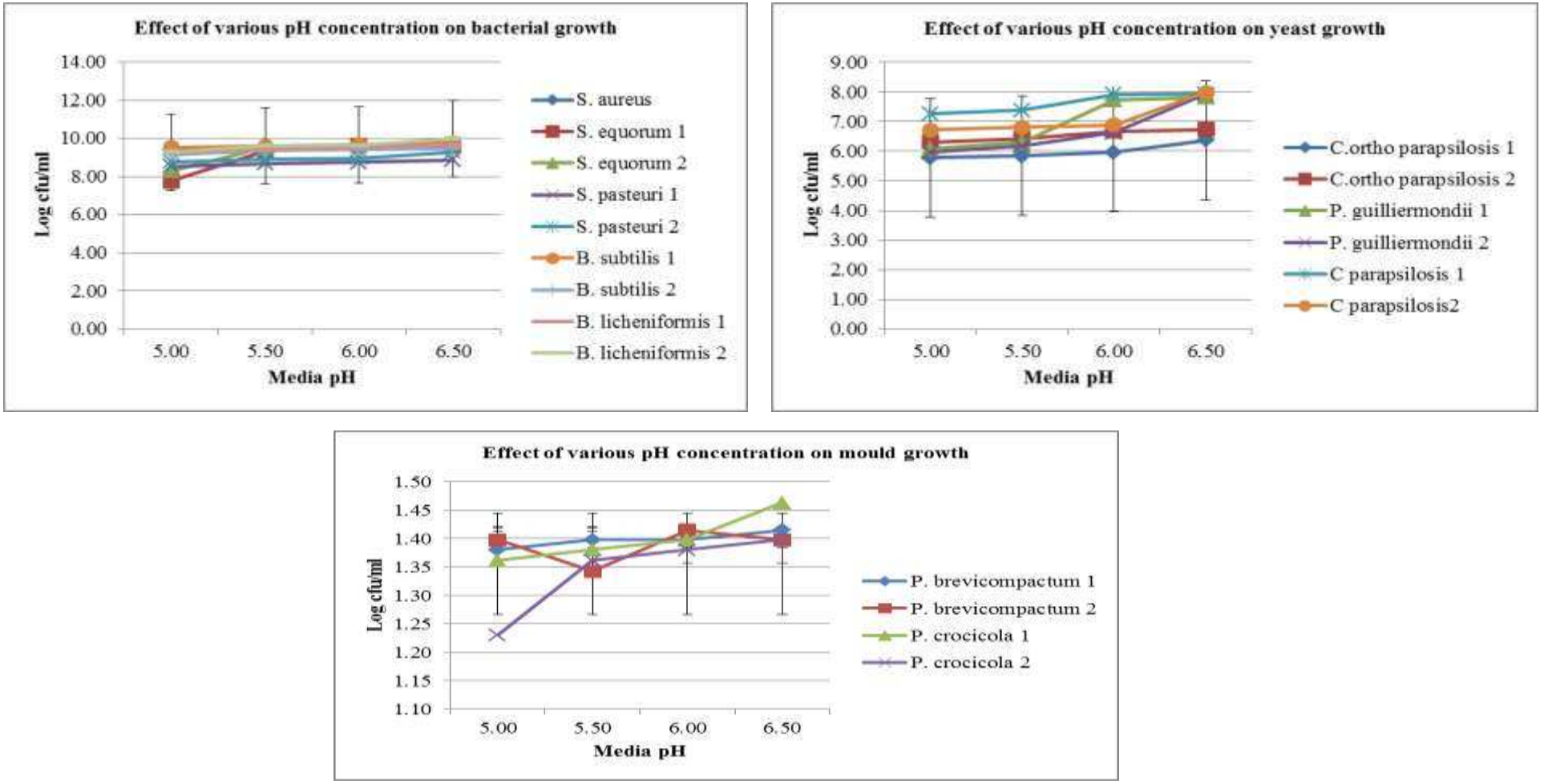


Figure 6.6 Effect of various pH on the growth rate of bacteria and Y&M.

The pH of the original spread varies from 6.5 to 6.7 with a target of 6.5. Therefore a laboratory scale spread production was performed at pH 5.0 to observe the level of microbial contaminants comparing to a standard production product. But, the acidity was really noticeable and changed the flavour organoleptically. No further testing was carried out, as this option did not seem a feasible approach to solve the problem.

6.2.4 Effect of rosemary herbs

Studies (Shelef et al., 1980; Shelef 1983) showed that the growth of both Gram positive and Gram negative foodborne bacteria, yeasts and moulds can be inhibited by herbs and spices. Rosemary in the form of the volatile oils contains antibacterial and antifungal properties; it is specifically active against Bacillus cereus and Staphylococcus aureus. The 41 components identified in the essential oil of rosemary include camphor, verbenone, 1.8-cineole and α-pinene; all have an effective antimicrobial power (Moghtader and Afzali 2009).

For this experiment Chapex-Dox, OGYEA and Nutrient Agar were prepared with 0.55g/l of rosemary herb, a concentration used by other dairy manufacturers identified by GC-MS method performed by Reading Scientific Services LTD. The data showing this are not presented due to confidentiality reasons.

The results are presented in Figure 6.7. S1 and S2 are two different strains of Staphylococcus equorum, S3 and S4: two strains of S. pasteuri; B1 and B2: two different strains of B. subtilis; B3 and B4: B. licheniformis. Y1/ Y2, Y3/Y4 and Y5/Y6 are two strains each of C. ortho parapsilosis, P. guilliermondii and C.

parapsilosis respectively. M1 and M2 are P. brevicompactum strains and P. crocicola are M3 and M4 strains. A control plate of NA was used in this experiment alongside the medium with 0.55g/l concentration of rosemary herb in order to compare the antimicrobial effect of the herb on the presumptive causative microbial agents. It was observed from that the total counts of each strain were not very different from the control. One log kill was not observed in any of the strains. The results show rosemary had no effect on the causative organisms

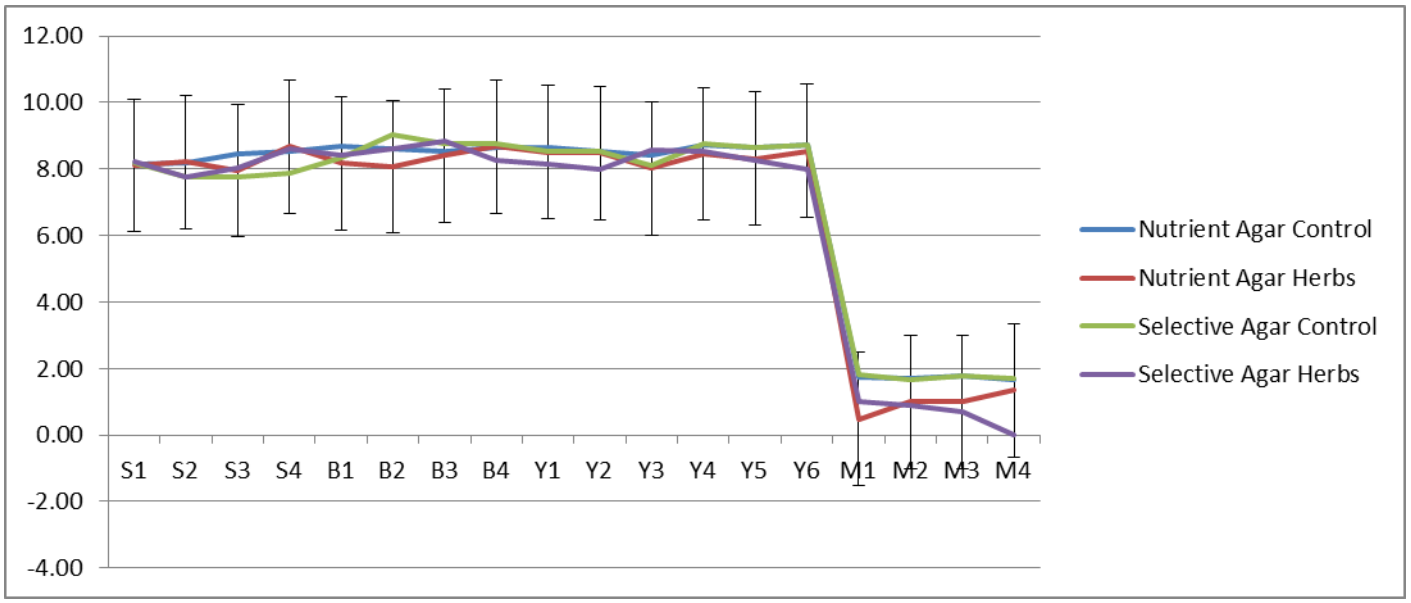


Figure 6.7 Effect on microorganism growth when compared with Nutrient Agar and Rosemary Herb supplement (0.55g/l) Nutrient Agar

6.3 DISCUSSION

Lipases (triacylglycerolacylhydrolases) are an ubiquitous enzyme occurring widely in bacteria, yeasts and moulds as Bacillus spp, Staphylococcus spp, Penicillium spp, Aspergillus spp, Rhizopus spp, Candida spp, Acinetobacter spp etc. (Momsia et.al., 2013). Lipase has been an integral part in the food processing industry. In the dairy sector they are used for flavours and ripening of cheese as they cause lipolysis of butter, fats and cream. The enzyme releases short chain FFA (C4 and C6) arising various flavours with the release of medium chain FFA (C12 and C14) (Arpigny et. el., 1999). The flavour attributed by these compounds range from pleasant fruity esters to putrid sulphur compounds (Engles 1996) that can be identified via gas chromatography – mass spectrometry (GC-MS). The catalytic properties of the α / β hydrolyses absorbs into the oil / water interface of an emulsion. These can also be dependent on the pH and temperatures. The most studied group of microorganisms for lipases are Bacillus spp, Staphylococcus spp and Pseudomonas spp. It has been noted that these enzymes can be heat stable at 77ºC with a holding time of 17 sec and 140ºC for 5 sec (Griffiths et. al., 1980 Gram positive and negative psychrotophs are able to grow in the refrigeration temperature ≤5ºC, majority of which are destroyed at pasteurisation temperature other than Bacillus spp (Griffiths et. al., 1980). But the extracellular enzyme as lipases are heat stable and are able to break down the milk fat at very low concentration over a longer storage time.

Methyl ketones primarily contribute to the mould ripened ‘cheese flavour’ (Engels et.al., 1997) attributed to 2-heptanone and 2-nonanone. They are formed in cheese by enzymic oxidative decarboxylation of fatty acid via the β carboxylation pathway in the cheese ripening environment. But the same flavour in spread is unwanted and denoted as a sign of spoilage due to the presence on microbial lipases that are temperature stable (Joseph et.al., 2008). Cold active lipases from psychotrophic microorganisms show high catalytic activity at low temperatures compared to the lipase enzyme isolated from mesophilic or thermophilic bacteria. Bacillus spp, Staphylococcus spp and YandM such as Aspergillus spp, Penicillium spp and Candida spp can all produce cold adapted lipases that are active ≤5ºC (Joseph et.al., 2008).

Abuse test results coupled with ingredient and product screening suggest that causative organisms are present in circa 63% of production and the causative organisms must be present for the development of the ‘cheese flavour’’ taint. Inoculation of presumptive causative microorganisms into ingredients has determined that the microbes utilise the hard stock element of the recipe i.e. oil blend or butter oil. As the microbes identified are either facultative anaerobes or strict aerobes their growth increases in the presence of oxygen so, once the pack is opened and the product is aerated, ‘cheese flavour’ development accelerates. Any temperature abuse caused by the consumer significantly increases the speed of ‘cheese flavour’ development. From the FSA funded Combase growth predictor (www.combase.com) it was noted that bacterial microorganisms’ doubling rate increases significantly at 10ºC compared to 5ºC as supported by the Chill Food

Act 1998. On the other hand bacterial doubling rate increases to every 30 min at ambient or at body temperature ~37ºC (Jay 1996). On the other hand YandM take significantly longer time to grow at ambient or chilled temperature compared to bacteria.

The particles in solutions and colloids are in constant motion. The manufacturing process strongly affects the rheological behaviour of the final product depending on the processing parameters as the cooling rate, shear and processing temperature that will affect the crystallisation process (Ronholt et.al, 2013). The commercial manufacturing methods include either churning or emulsion technology. The churning technology is based on precrystallisation of cream and can be both batch and continuous process.

The scraped surface heat exchanger (SSHE) is a jacketed cylinder having a rotating dasher with rows of scraper blades. The product is pumped through the cylinder as the heating or cooling medium is circulated into the cylinder. The heating medium can be steam, water or cooling medium a refrigerant such as ammonia or Freonn etc. (Harrod, 1986). The droplet size of the emulsion is primarily determined by the intensity of flow around the scraper blades, typically producing a droplet size of 5µ in margarine. Water, or a cooling fluid, is flowed through the cavity of the jacket during emulsification. The combination of cooling and shearing is necessary to promote the formation of fat crystals. It was found in Section 5 that the processing under SSHE, the trial products were more robust against the growth of the causative organisms giving a ‘cheese flavour’’ spoilage

aroma. Based on the new and improved processing method, the product formulation was further challenged here by looking at various antimicrobial agents to be built into the product recipe as potassium sorbate (0 to 0.2%), salt acidity (pH 5.0 – 6.5) and rosemary herbs (0.55 g/l). The main antimicrobial agent of focus was potassium sorbate / pH when compared against the market competitor products and the herb for a range extension product with added flavour.

All microorganisms have an optimum growth conditions in a set of environment parameters. The growth rate and survivability is affected outside of this range (Jay 1996). Under extreme conditions the bacteria can undergo stress. Microorganisms have adaptability to respond to non- lethal tress conditions and can results in enhanced resistance. Microorganism's adaptability in low pH condition is known as 'Acid Tolerance Response (ATR) (Dodd 2005). This is influenced by variety of factors as type of acid (weak / organic acids), pH, growth phase etc. the Acid Shock proteins (ASPs) regulated due to ATR induce cellular metabolism or repair functions by regulating the global stress response rpoS (σ^s) (Garner 2006).

Potassium sorbate is a common food preservative but the antimicrobial activity is highly pH dependant at the level of ≤4.5 (Dodd 2005). In this section while altering the product formulation, the sensory affect was also evaluated by a triangle test. Current recipe of Spread A contains neutral pH and lowering the level to ≤ 5.5 changes the product sensory profile and it wasnot a move that was advisable.

Minimum lethal concentrations (MLC) of rosemary were evaluated against Listeria monocytogenes, Staphylococcus aureus, Escherichia coli O: 157:H7, Yersinia enterocolitica, Pseudomonas aeruginosa, Lactobacillus plantarum, Aspergillus niger, Geotrichum, and Rhodotorula. (Elgayyar et al., 2001) Rosemary oil was not particularly inhibitory to bacteria (inhibition zone, 25 mm); however, it was highly inhibitory to moulds. The minimum inhibitory concentration of rosemary against the Gram positive strains are between 2 and 15 μg/ml, Gram negative bacteria between 2 and 60 μg/ml and for yeasts and moulds 4 μg/ml (Abramovic et.al., 2012). Rosemary and several of its constituents, including carnosic acid and carnosol, have exhibited antibacterial effects against various Gram-positive and Gram-negative bacteria in vitro including oral planktonic bacteria Bacillus subtilis, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, methicillin-resistant Staphylococcus aureus (MRSA), lactobacilli, Listeria monocytogenes, Streptococcus thermophilus, Pseudomonas fluorescens, and Yersinia enterocolitica (Abramovic et.al., 2012).

One of the limitations regarding the study of the mould strains was that these could not be inoculated at the same level as bacterial or yeast strains due to the mycelium production.

Looking into the FSA regulation on sodium salt concentration, the next chapter was focused into challenge testing on Listeria monocytogenes, a dairy pathogen of concern on various other salt levels to be used as a suitable replacement.

CHAPTER 7 MICROBIAL CHALLENGE TEST

The study in this Section was focused into challenging Listeria spp at various dairy environment conditions and different spread formulation to determine the hurdle against the organism within the industry.

The Listeria strains used in this study were isolated from an external laboratory service provider organism bank. They were positives identified while sampling various dairy environments and processing areas. The name of the lab and the source of the strains were kept anonymous due to confidential reason, a total of six different sources were used in the study. These strains were used to perform challenge at different processing conditions as the pasteurisation or CIP to identify if the bacteria will be able to survive if introduced to the plant at various processing stages. Figure 7.1 illustrates the mode of transfer of the organism from the environment to the product.

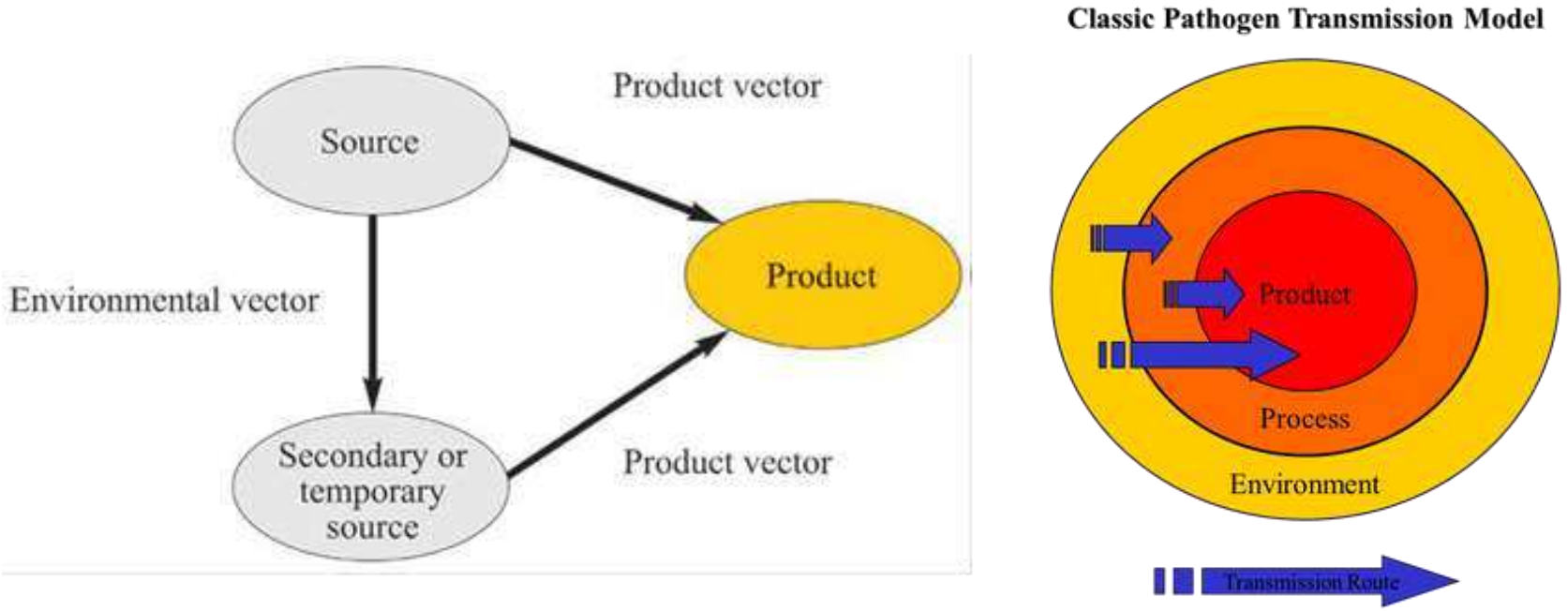


Figure 7.1Transfer of pathogens from sources to food / product via ingredients / products / vector / indirectly or directly

(Holah et.al, 1993).

7.1 LISTERIA SPP ISOLATION

The manufacturing companies as part of their GMP and GHP frequently swab the process environment including both direct (fillers) and indirect food contact (drains, floor, walls, conveyors) along with finished product to comply with EU Micro Criteria 2073/2005. The strains from the laboratory bank were isolated from environmental swabs (Section 3.3.4) and analysed to isolate Listeria spp as per method 3.1.3.6. The isolated strains were sent out to an external laboratory (Campden BRI) for ribotyping.

A typical environmental swabbing plan as per Codex Alimentarius Commission 57-2004 was carried out across the factory covering a minimum of $10cm^2$ surface areas (Method 3.3.6.1). The isolated strains were identified in a routine monitoring including areas as filler room, filler heads, floors, drains, conveyors, raw milk silos, finished milk tanks, samples of the pasteuriser outlet and finished packed product a three months period. A general dairy process can be segregated into four Sections raw (raw milk intake & storage as raw milk silo (RMS) & reclaim milk tank (RCT), process (pasteurisation (PA) and storage of intermediate product as finished milk tank (FMT), filling hall and lastly coldstore and despatch. These areas can be physically segregated from each other. Raw milk is stored in the RMS and is pumped under positive pressure to the pasteuriser (PA) and processed at 72.5ºC for 25secs and stored in FMT for intermediate storage. The milk is further pumped under positive pressure to the fillers. Once packed the bottles transfer to the cold store through a conveyor system and are packed at the end. The factory wastes are usually taken toa compactor site through an airlock

system. The engineering work shop may be an integral part of the factory or just outside of the main production area with restricted access following GHP. Figure 4.2 illustrates a typical dairy process and product flow in the factory.
The isolated Listeria spp were further grouped into 6 categories based on the source. Table 10.4 and Figure 10.2 in Appendix 10.7 shows the % similarity between the identified strains via Ribotyping carried out at Campden BRI. The Ribotyping of strains showed that the isolates from the same source had similar genetic profile (Figure 10.2). Source A, B, D and E contained L. monocytogenes whereas source C had only L. innocua and F had a mixture of L. innocua and L. monocytogenes species.

After analysing the data in Table 10.4 it was noted that genetically identical strains (≥ 95%) were isolated from source A where the swab points included the filler environment, labeller, conveyor, and accumulation table prior to the labeller and various floor areas between the ends of line Packers.

A second strain of Listeria monocytogenes (source B) was isolated from raw milk silos, milk storage tanks etc. The contaminant may have transmitted through trolleys, and general traffic flow. The strain in the storage tanks may also be a result of environmental contamination. Listeria monocytogenes strains were identified in the raw milk silo, which is very normal. The positive samples from the milk tank may bedue tocross contamination through the operators while sampling. This strain showed less than 86% similarity to the Listeria monocytogenes strain in source A showing they are different.

The rest of the isolates were genetically different L. monocytogenes and L. innocua strains. Genetically identical L. innocua strains were isolated from the engineering changing rooms, airlock near the compactor and various floor areas. The spread of L. innocua strains may be through the changing room and the compactor area and also by the people flow route. The presence of L. monocytogenes strains may follow the same hypothesis.

7.2 LISTERIA SPP CHALLENGE TEST

Challenge testing helps to assess the potential for growth of a specific microorganism in a food product from the point of manufacture through to customer consumption. This was done by inoculating a product with a known level of Listeria spp and testing under a range of controlled environmental conditions in order to assess the risk of either food poisoning or to establish a product's stability during shelf life.

7.2.1 Listeria monocytogenes challenge test in cream

The purpose of this study was to inoculate cream with a known inoculum of L. monocytogenes at three different levels 10, 50 and 10^2 cfu/ml for three separate trials. The organisms used were wild strains of Listeria monocytogenes isolated from source A as in Section 7.1. An NCTC strain 11994 of L. monocytogenes was used as a control. The study was carried out in cream over 16 days replicating

fresh milk and cream shelf life in the industry. Standard shelf life of fresh milk and cream in the market is 10 to 12 days. The challenge test was done upto 16 days to consider the consumer abuse and also as a part of due diligence for food safety and quality. The Listeria monocytogenes inocula for the challenge testing were prepared as per Sections 3.3.6.2 and 3.3.6.3.

The inoculated samples were stored at 8ºC over 16 days to follow the conditions used in the retail chill chain and screened for TVC (§3.1.2), Enterobacteriaceae (Ent) (§3.1.3.1), Lactic Acid Bacteria (LAB) (§3.1.3.10), pH (§3.2.1) and aW (§3.2.1.7) from the day of inoculation. All these tests were performed at Day 0 and 16 to understand the general level of contamination in the original sample and if it would have created a competition with the L. monocytogenes and affected the growth rate. A control sample of sterile milk (autoclaved at 121ºC for 15 mins) was incubated along with the inoculated sample and screened for TVC, LAB, Ent and L. monocytogenes on Day-0 and 16. For L. monocytogenes were screened on days 6, 12 and 16 respectively to evaluate the growth rate.

Table 7.1 shows that in all three challenge tests the Maximum Growth Potential (δ) was >0.5 log, therefore classifying the product as a "Ready-to-eat food able to support the growth of Listeria monocytogenes other than those intended for infants and for special medical purposes" (EU Micro Criteria 2073:2005). The growth potential (δ) is the difference between the $\log_{10}$ cfu/g at the end of the test and the $\log_{10}$ cfu/g at the beginning of the test. The experimental results show a

wide dispersion, notably because the lag phase was included. As shown in Figure 7.2, the growth potential in Challenge tests 1, 2 and 3 were 7.54, 7.33 and 6.96 respectively, showing the product is able to support the growth of Listeria in milk over a 16 day product shelf life with a similar food matrix as milk or cream. The 16 day shelf life was based on the industry standard for fresh milk shelf life of 12 days and cream 14 days.

Table 7.1 Listeria monocytogenes challenge test in cream

Experiment	Sampling Day	Count log cfu /ml					pH	aW	Growth Potential
		Control Samples (cfu/ml)	*L. mono*	TVC	LAB	Ent			
1	0	0	0.30	2.54	0.90	0.30	6.74	0.98	7.41
	6	0	2.53						
	12	0	3.41						
	16	0	7.71	5.45	1.08	2.54	6.65	0.98	
2	0	0	0.00	2.76	0.00	0.00	6.73	0.98	7.49
	6	0	2.13						
	12	0	3.32						
	16	0	7.49	5.54	1.18	2.08	6.59	0.98	
3	0	0	0.30	2.00	0.00	0.00	6.7	0.98	6.08
	6	0	3.03						
	12	0	3.46						
	16	0	6.38	6.08	1.32	2.30	6.65	0.98	

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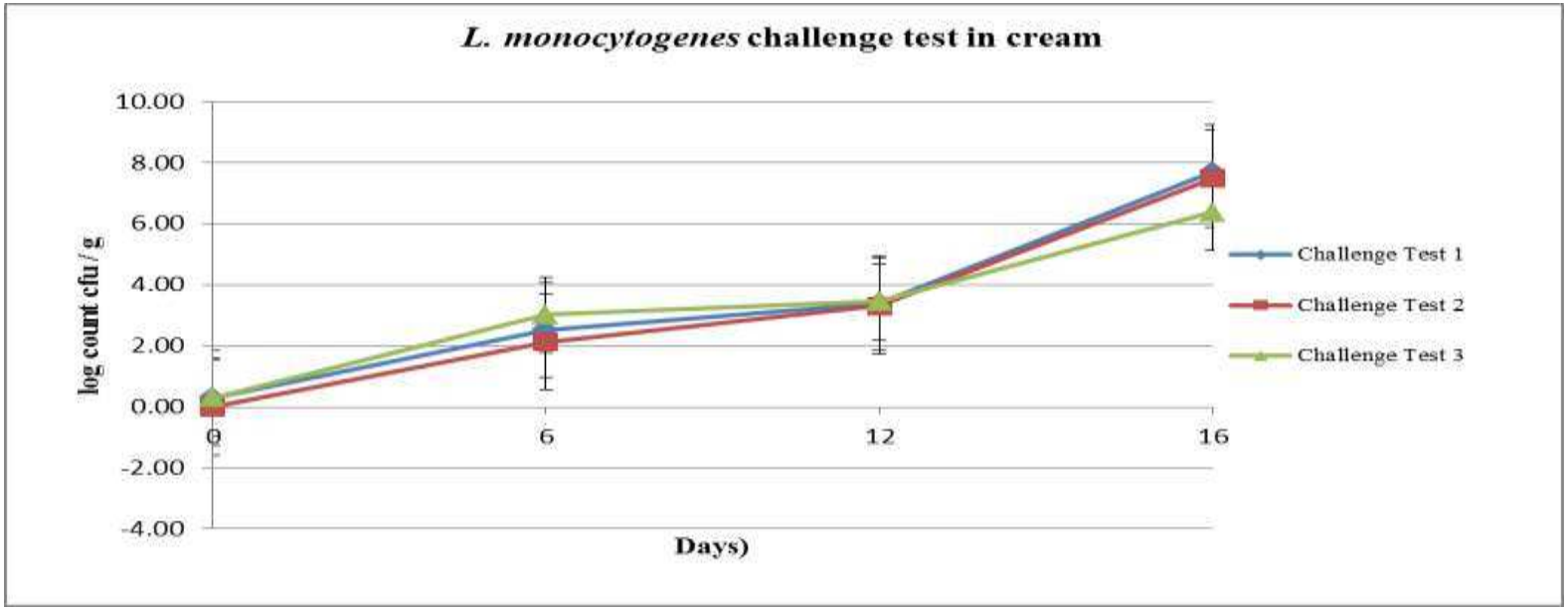


Figure 7.2 Listeria monocytogenes spp challenge test in cream

7.2.2 Listeria monocytogenes challenge test in emulsion

The stab method was used for challenge testing (Voysey et al. 2009). All the identified Listeria monocytogenes in source A and B (Table10.4) were prepared as per Section 3.3.1 for further challenge testing. An NCTC strain 11994 of L. monocytogenes was used as a control. One colony was stabbed into 10 g of a sterile emulsion sample collected from the factory processing and autoclaved at 121ºC for 15 secs and inoculated as per Section 3.3.3 (Holliday and Beuchat 2003). This method does not disturb the product structure, and also avoids introduction of extra water.

Both microbiology and physico-chemical properties of the product were analysed to ensure that the batches of product used in the challenge test were reflective of the products produced during routine manufacture. The data presented here are only the growth rate of L. monocytogenes under various chemical parameters. The test was carried out to determine the effect on L. monocytogenes growth in product under various intrinsic parameters and if any had antibacterial affect while keeping the product colour, texture and taste the same.

Figure 7.3 shows the L. monocytogenes growth pattern in emulsions at 1.5% salt and at variable pH of 5.5, 6.0 and 6.5. pH 5.5 had a slightly slower growth rate, 4.26 log cells / g in 113 hours compared to 4.99 and 5.46 log cells/g at pH 6.0 and 6.5 respectively. Hence the use of citric acid to lower the pH to 5.5 from the

current neutral recipe (Table 5.2) of 6.0 would slow the growth of the organism if it is already present as a contaminant.

Figure 7.4 shows the L. monocytogenes spp growth pattern in emulsions at pH 5.5 and at variable salt levels 0, 0.5, 1.0 and 1.5% NaCl. The graph shows the growth was comparatively slower at 0% salt due to absence of nutrients compared to the other three different concentrations. This shows to achieve the current FSA salt target for 2017 (1.48% for salted butter, 1.13% slightly salted and 1.06% for Spread) (FSA 2014), no extra hurdle for the bacterial growth will be achieved at 1.5% compared to 0.5%.

The growth pattern at 0.95 a_W and at variable pH of 5.5, 6.0 and 6.5 (Figure 7.5) showed similar growth pattern as for 1.5% salt at same pH variable range (Figure 7.3). The bacterial growth rate (μ) was 0.0095 log cfu/ml at pH 5.5 compared to 0.0122 and 0.0141 log cfu/ml at pH 6.0 and 6.5. Thus acidic pH slows the growth of the contaminant.

Figure 7.6 illustrates the L. monocytogenes growth pattern at 0.93, 0.95 and 0.98 aW and pH 5.5 showing similar growth characteristics as in Figure 7.5 where growth rate μ at pH 5.5 and aW 0.95 were 0.0095 log cfu/ml and a longer lag phase was observed.

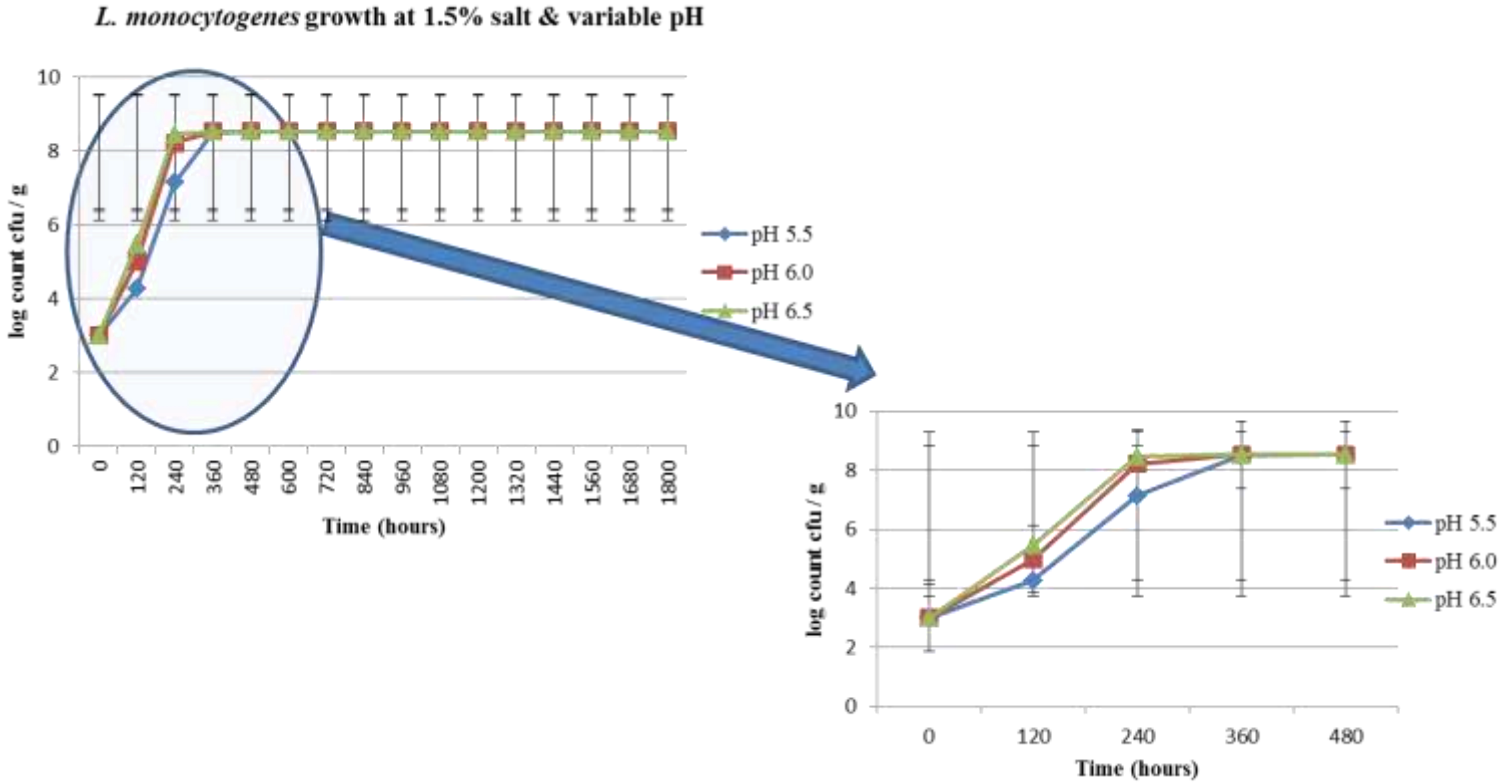


Figure 7.3 Listeria monocytogenes growth pattern in emulsion with added 1.5% salt and at variable pH: 5.5, 6.0 and 6.5.

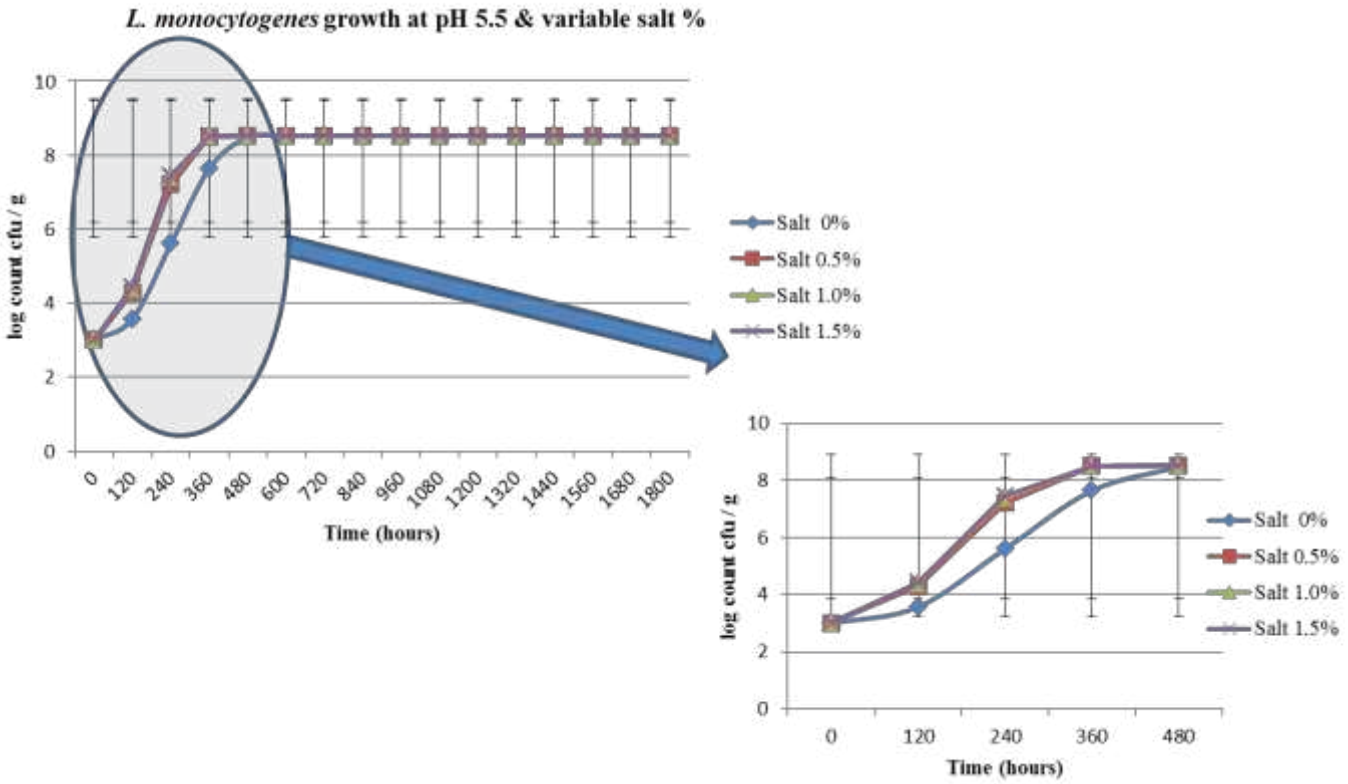


Figure 7.4 Listeria monocytogenes growth pattern in emulsion at pH 5.5 and at variable salt levels 0.0, 0.5, 1.0 and 1.5%.

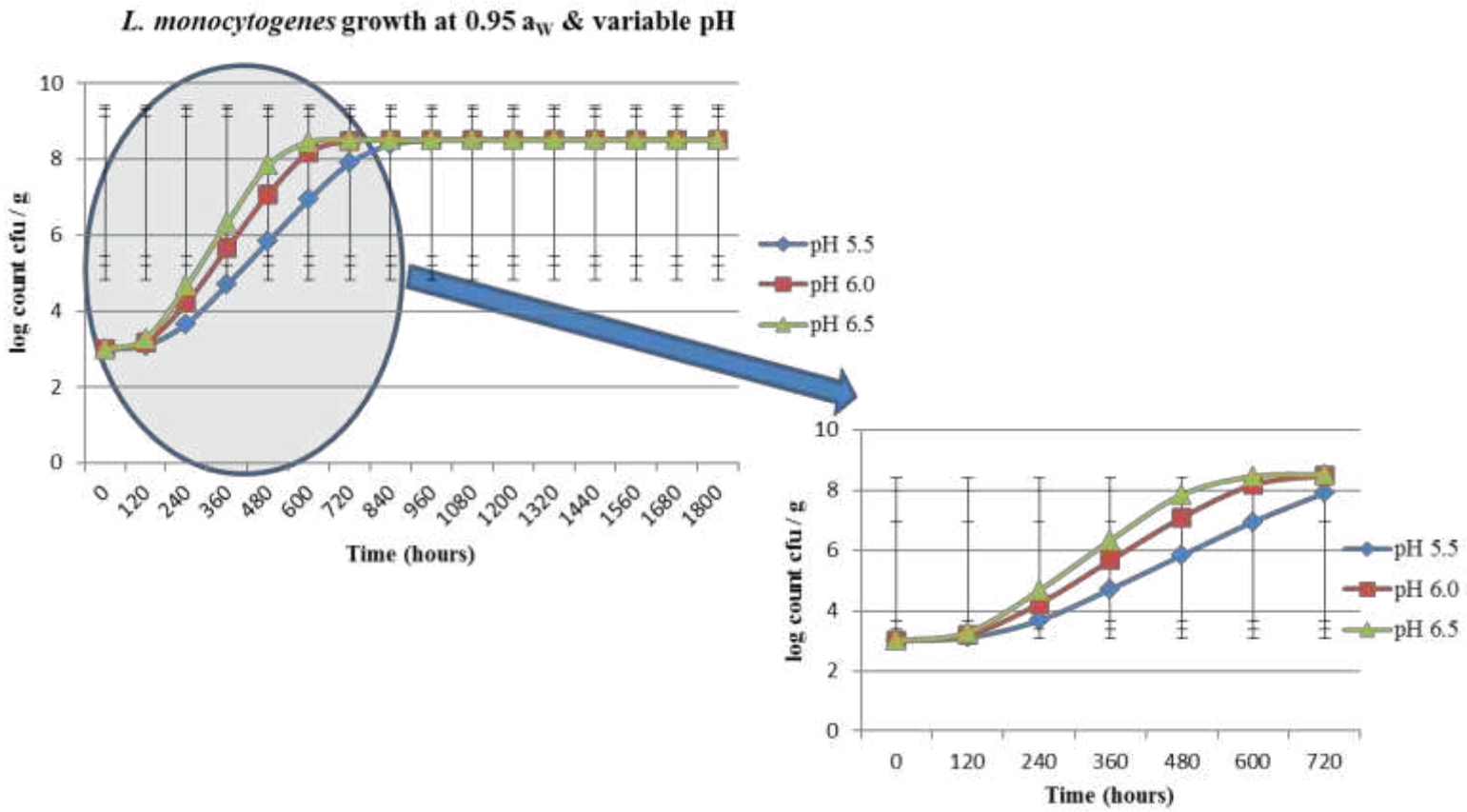


Figure 7.5 Listeria monocytogenes growth pattern in emulsion with 0.95 water activity at variable pH: 5.5, 6.0 and 6.5.

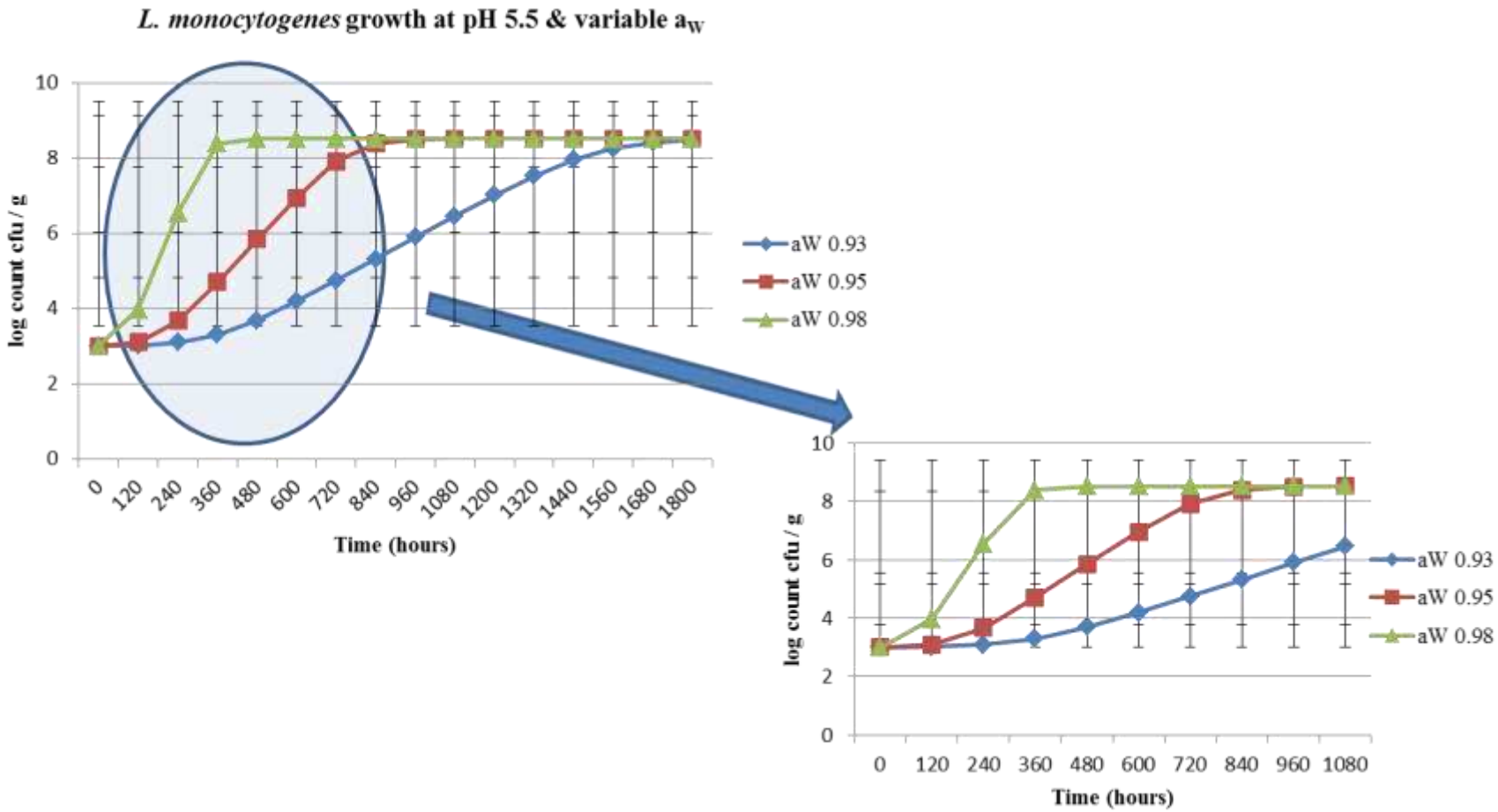


Figure 7.6 Listeria monocytogenes growth pattern in emulsion with pH 5.5 with variable a_W 0.93, 0.95 and 0.98.

7.2.3 Listeria spp challenge test in spreads

The challenge tests performed in Section 7.2.2 were in the emulsion state of the spread processing stage where the samples were taking off the working Section after the churn prior to the scrape surface heat exchange step (SSHE).

To further understand the product matrix , a challenge test was performed with Listeria monocytogenes in the finished product after it had undergone all processing steps An external service provider Campden BRI was used for product due diligence purpose. The challenge test was designed to ascertain whether the organisms would be capable of growth within the spreads during the shelf life of products. The protocol for the challenge test was based on methods developed in an FSA funded project on the survival of Listeria in butter (FSA 2008).

The inoculum was prepared and inoculated into the sample as per Section 3.3.6.5. The Listeria monocytogenes challenge test in spread (Figure 7.7) shows that the Listeria average inoculum remained relatively constant within the 13-week testing period. (DHB –STM is the product code sent to Campden).

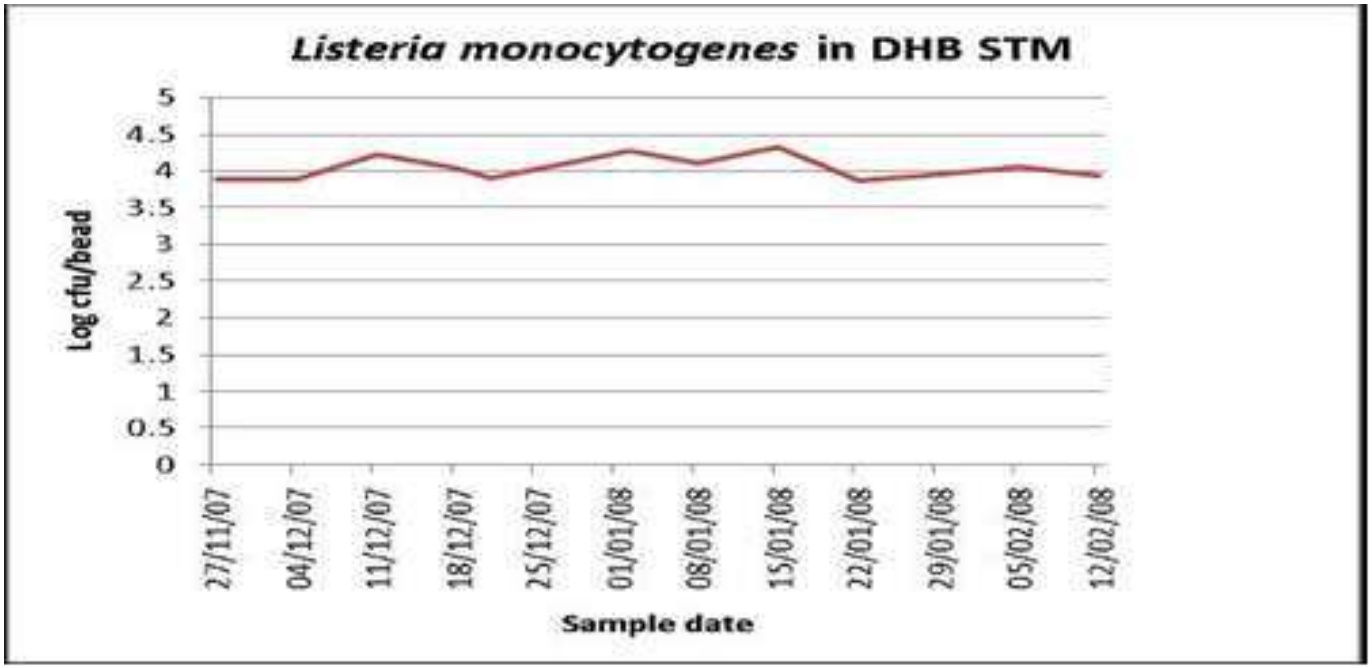


Figure 7.7: Listeria monocytogenes challenge test in spread.

In each of the inoculated spreads no growth of Listeria monocytogenes was observed over the challenge period although in the majority of cases the organism was capable of survival. No Listeria spp was detected in the un-inoculated control samples during the challenge test duration; although a low level of Bacillus licheniformis did develop by week ten. The challenge test therefore demonstrated that L.monocytogenes was not capable of growth within the test period in each of the products.

7.2.4 Listeria spp heat resistance test

The study was aimed at assessing the heat resistance of wild strains of Listeria monocytogenes isolated from source A and B, even though Listeria spp is not generally considered to be particularly heat resistant. Several studies have demonstrated inactivation of the organism by pasteurisation (72ºC for 15 seconds or equivalent heat treatments) used for commercial milk pasteurisation (Jay 1996; Sprenger 1998).

L. monocytogenes isolates were inoculated into milk samples which were exposed to one of the following heat treatments (only L. monocytogenes was used in this experiment instead of both L. monocytogenes and L. innocua as the former is a known pathogen for humans and EU legislation is more focused on L. monocytogenes) (Section 3.3.6.5):

- 65ºC held for 25 seconds (pasteurisation)
- 72 ºC held for 25 seconds (dairy industry standard)
- 73.6 ºC held until the temperature was reached (pasteurisation divert)
- 78 ºC held for 25 seconds (company standard)

The first two sets of time / temperature are industry standard pasteurisation conditions. The third set is the divert temperature set for the dairy pasteuriser and the fourth one is the actual pasteurisation temperature at a dairy processing site.

None of the temperature-exposed (as above) organisms exhibited signs of growth of L. monocytogenes as determined per Section 3.1.3.6 under the ISO method, general culture method and VIDAS. It can therefore be concluded that the two strains of L. monocytogenes were not resistant to the heat treatments applied. This highlights the contamination was more likely to be post process rather than transmission through the plant itself (data not presented).

7.2.5 Listeria spp acid and alkali resistance test

The Purpose of CIP (In-Place Cleaning) is to provide an acceptable degree of cleanliness in equipment without a significant amount of dismantling. In order to obtain the optimum effectiveness from CIP, the design and construction of the plant that is required to be cleaned must be studied as carefully as any equipment that supplies cleaning fluids for CIP (a CIP "unit" or "set"). The CIP sequence usually follows the sequence of pre-rinse, detergent circulation (min. 70ºC),

intermediate rinse to remove residual detergent, sanitiser circulation and final rinse with potable water (where necessary to remove non-food compatible disinfectant). An additional acid circulation and rinse can be used where needed to remove stubborn inorganic deposits and scale. This is a standard procedure across the food industry.

The aim of the study was to assess if wild strains of Listeria monocytogenes were resistant to the following environmental conditions a typical detergent was and acid was steps were followed along with an extra acid wash steps:

- 0.8% alkali (NaOH) solution held at 80ºC for 40 minutes (Detergent)
- 0.6% acid solution (HCl) held at 50ºC for 12 hours (Acid Wash)
- 0.6% acid (HCl) solution held at 50 ºC for 24 hours (Acid Wash)

The experiments in this section were carried out to understand the resistance of the isolated specific strains to the factory CIP (Clean in Place) system of acid and caustic treatment cycle.

After inoculation an assessment of the L. monocytogenes colony number was made by plating out onto both selective and non-selective media. A high target level of inoculant (L. monocytogenes cells) of approximately $10^7 – 10^8$ cfu/ml was used for this study. After inoculation, the pH of the broths was altered by adding the required volume of HCl or NaOH whilst constantly mixing the broth to create the 0.8% and 0.6% solutions. Dehydrated culture (Section 3.3.6.7) as well as fresh cultures was used here to understand the kill effect on both as the organism

can attach to the processing equipment in a dehydrated state to adapt to the harsh environment. The inoculated broths were treated for the following durations and temperatures:

- 0.8% NaOH solution – both the fresh cultures and dehydrated cultures were incubated at 80ºC for 40 minutes
- 0.6% HCL solution – 1 broth of fresh culture and 1 broth of dehydrated culture were incubated at 50ºC for 12 hours and 0.6% HCL solution - 1 broth of fresh culture and 1 broth of dehydrated culture were incubated at 50ºC for 24 hours. After the heat treatment samples were rapidly cooled to room temperature before being analysed for Listeria spp for detection and enumeration with the ISO method (Section 3.1.3.4).

For both the fresh and dehydrated culture samples treated with 0.8% NaOH and held at 80ºC for 40 minutes, the enumeration results obtained on chromogenic media were ≤1cfu/ml. In addition a non-selective medium was also used to facilitate recovery of sub-lethally stressed cells which may not have been capable of growth on the chromogenic media; a result of ≤1cfu/ml was also obtained from this medium. For the culture samples treated with 0.6% HCl and incubated for 12 hours at 50ºC the results in Figure 7.8 were obtained on chromogenic agar. Samples treated with 0.8% NaOH at 80ºC for 40 minutes and 0.6% HCl at 50 ºC for 24 hours did not show the presence of any Listeria spp in the presence/ absence detection method. But samples treated with 0.6% HCl at 50ºC for 12 hours showed the presence of the strain.

There are currently three different methods available for the presence absence test of Listeria spp in food. They are the traditional Culture method, STD ISO method and VIDAS as per Section 3.1.3.6. Listeria monocytogenes was isolated using three different methods in order to validate the sensitivity of the methods. The organisms were enumerated as per ISO 10560:1993. In this experiment all three different methods were used as according to Waak et al. (1999) the ISO method is more sensitive than the culture method to understand the recovery rate for all different methods.

All samples analysed by the culture method (ISO 10560:1993) (3.1.3.6) showed 'Not Detected' in 25ml. Detection for Listeria monocytogenes with the VIDAS method (3.1.3.6) (performed at an external laboratory) with samples treated in 0.8% NaOH were also 'Not Detected' in 25ml. Samples treated with 0.6% HCl and incubated for 12 hours showed a result of presumptive detected in 25ml result. Treatment with 0.6% HCl and incubated for 24 hours were also 'Not Detected' in 25ml. Detection under the ISO method (BS EN ISO 11290-1:1997, BS 5763-18: 1997) also produced negative results as shown in Table 7.2. This indicates the isolated strains were not resistant to the acid / alkali treatment or the CIP (Clean in Place) system of the factory.

Sufficient data was not available for a conclusive answer as to whether the ISO method was more sensitive than the VIDAS or culture methods (Table 7.2). Figure 7.8 shows the Listeria chromogenic agar with the two highest counts for both fresh and dehydrated samples for the 12 hour HCl treated culture. Picture A on the left is for fresh and B on the right for dehydrated cultures. Fresh samples

gave counts of 60 cfu/ml and dehydrated samples 6 cfu/ml. Colonies are typical of Listeria monocytogenes.

Table 7.2 L. monocytogenes resistance to acid and alkali treatment

Type of culture	cfu / ml	Method of detection in 25 ml		
	Enumeration	Culture	VIDAS	ISO
Test Method	0.8% NaOH solution results summary:			
Fresh	≤1	ND	ND	ND
Dehydrated	≤1	ND	ND	ND
Test Method	0.6% 12 hour HCL solution results summary			
Fresh	60	ND	Presumptive, unable to grow in OAA positive	ND
Dehydrated	6	ND	Presumptive, unable to grow in OAA positive	ND
Test Method	0.6% 24 hours HCL solution results summary			
Fresh	≤1	ND	ND	ND
Dehydrated	≤1	ND	ND	ND

A

B

Figure 7.8 Listeria chromogenic agar (Picture A on the left is for fresh and B on the right for dehydrated culture.)

7.3 LISTERIA SPP GROWTH CURVE

The main ingredient for spread making is cream, which is a bi-product in a milk factory. Listeria spp can grow (Fain et al. 1991) in milk and cream and may also be able to survive or multiply in a low salt spread product. The main product hurdle therefore will be the product droplet size. Hence a few challenge tests were carried out in order to understand the robustness of the product, process and bacterial strains that are all specifically from the dairy environment. All the experiments were repeated twice with three replicates to show the repeatability and reproducibility of the data. In all cases no significant differences or changes were observed. Tecan measurement, using Tecan GENios Pro which is a multifunctional microplate reader that can read a plate of 96 wells over a period of time to produce an automated growth curve was also performed simultaneously along with the manual growth curve. For the Tecan growth curve each well was inoculated with 200µl of the final volume of the mixture of broth and the bacteria and incubated at 30ºC for 24 h; each individual well was read every 30 minutes with a 5 seconds shake at 100 rpm prior to reading the optical density at 600 nm. Averages of all the experiments are presented here to give more accurate data, followed by the raw data with error bars of the replicate data in Appendix 10.7.

7.3.1 Listeria spp growth curve in broth

Listeria monocytogenes was inoculated at 1 cfu/ml volume concentration in 250ml of the Milk Plate Count Broth and incubated in a shaking incubator

(Sartorius model) at 300 rpm at 30°C and an optical density was measured every 30 minutes (§3.3.6.8). Source A (G1) and B (G2) L. monocytogenes were also grown in the liquid phase of the spread (Section 3 Table 3.4) which contains potassium sorbate at 0.063%. The raw data are presented in Table 10.5 (Appendix10.7) and an average growth rate was used in Figure 7.9. Figure 7.9 shows that the identified L.monocytogenes G1 and G2 had a significant growth rate in the MPC broth that is nutrient enriched compared to the minimal nutrient unsalted liquid phase of the spread. On the other hand the antibacterial effect of potassium sorbate was found to be significant in the unsalted liquid phase at 0.06% compared to the effect at 0.05% as per Section 6.2.1. In this experiment neither of the identified strains showed any growth at 0.063% of potassium sorbate that was identified as one of the antimicrobial agent in Section 6.2.1.

7.3.2 Effect of various salt levels on Listeria spp growth rates

According to Voysey et al. (2006), Listeria spp can grow in products with salt concentrations at more than 2%. As a result the industry is currently looking into salt replacements that will not affect the taste or flavour of the product but will give an extra hurdle rate for microbiological (pathogens / spoilage) growth. Listeria monocytogenes strains isolated from the dairy processing environment and products were challenge tested for various salt tolerances at different levels. Sodium chloride (NaCl), potassium chloride (KCl) and calcium chloride ($CaCl_2$) were used at the levels of 0.0, 0.5, 1.0, 1.5 and 2.0%. Milk Plate Count Broth pH 6.9 ±0.1 (Section 3 Table 3.3) was used for this experiment.

7.3.2.1 Effect of various salt levels on Listeria monocytogenes growth rates in broth

From Figure 7.10 it was observed that strain G1 gives similar growth patterns at NaCl (A) and KCl (B) levels from 0% to 2.0%. The growth is slightly reduced at 3% but this level is beyond the permissible level which can be legally added to products and so was not a useful hurdle to consider. On the other hand, the growth curve with $CaCl_2$ (Figure 7.10C) was different from that of A and B for all the salt levels of 0.5, 1.0, 1.5, 2.0 and 3%. The high initial optical density which shows a decrease over the first 5-7 hours may be as a consequence of the formation of an insoluble calcium sulphate salt. The composition of the broth contains sulphate ions that would react with the calcium chloride to produce calcium sulphate which in the course of time would settle out. After the salt settlement stage at 10 h the growth curve was very similar to that for the other two salts. Similar growth patterns for L. monocytogenes strains G2 were observed as shown in Figure 7.11. The raw data is presented in Appendix 10.7, Table 10.6 (G1 NaCl), Table 10.7 (G1 KCl) and Table 10.8 (G1 $CaCl_2$) and Table 10.9 (G2 NaCl), Table 10.10 (G2 KCl) and Table 10.11 (G2 $CaCl_2$).

For the experiments in 0% and 3% salt only a duplicate test were performed on each strains each salt type as the former was used as a control and the latter option is not aligned with the FSA salt target. The 3% salt concentration was used to observe the growth pattern. In the other concentrations at 0.5, 1.0, 1.5 and 2.0% salts the tests were performed as for replicates for each strain G1 and G2 L.

monocytogenes. Similar growth pattern was observed in each replicates per strain (Figure 10.3 (G1) and 10.4 (G2) in Appendix 10.7).

7.3.2.2 Effect of various salt levels on Listeria monocytogenes growth rates in liquid phase

Figure 7.12 A and B shows the growth of strains G1 and G2 L. monocytogenes strains were grown with three different salts (NaCl, KCl and $CaCl_2$ at various concentrations as per method 3.3.6.8 with added potassium sorbate as an antimicrobial at 0.06% concentration to the liquid phase (Table 3.6). The G1 and G2 strains didn't show any significant growth over a 24-h growth period. The experiment was repeated three times with four replicates of each salt type and concentration (the average raw data is presented in Appendix 10.7 Table 10.12 (G1) and Table 10.13 (G2). As no growth was observed, the process was repeated again but with no potassium sorbate in the liquid phase to understand its antimicrobial effect.

Figure 7.13 illustrates the growth rate of strains G1 (Table 10.14 Appendix 10.7) and G2 (Table 10.15 Appendix 10.7). It was observed that the growth pattern was very similar to Figures 7.10 and 7.11 where the MPC broth did not contain any potassium sorbate. The bacteria grew at all different salt types from 0% to 2% but the lag phase was significantly higher, nearly 15 h, compared to that in Section 7.3.2.1 where the study looked into the effect of the growth of L. monocytogenes in NaCl, KCl and $CaCl_2$ in MPC broth (Figure 7.10 and 7.11), which gave a lag phase of 5h.

A B

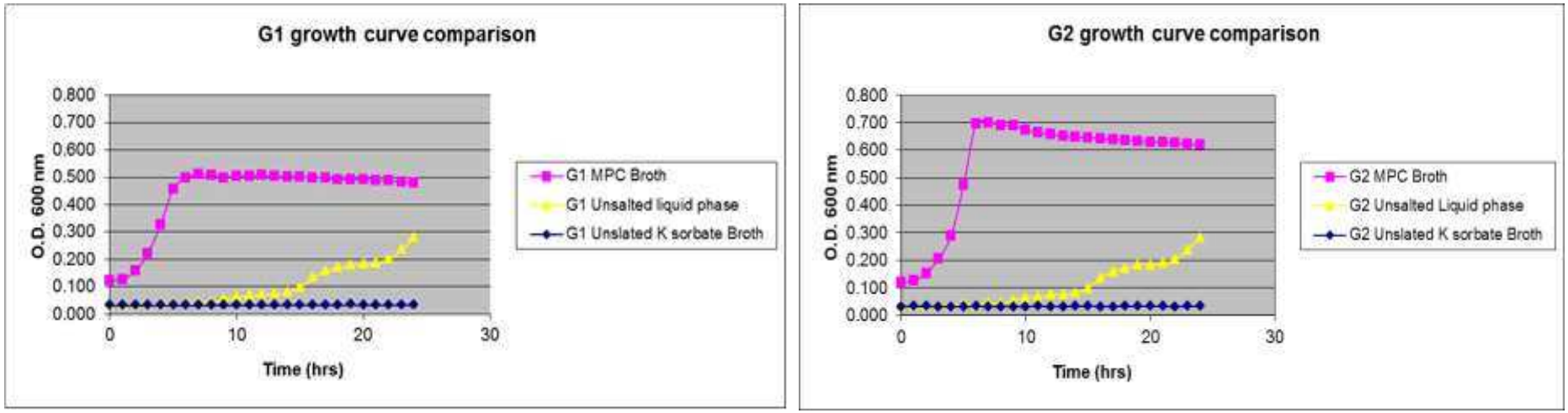
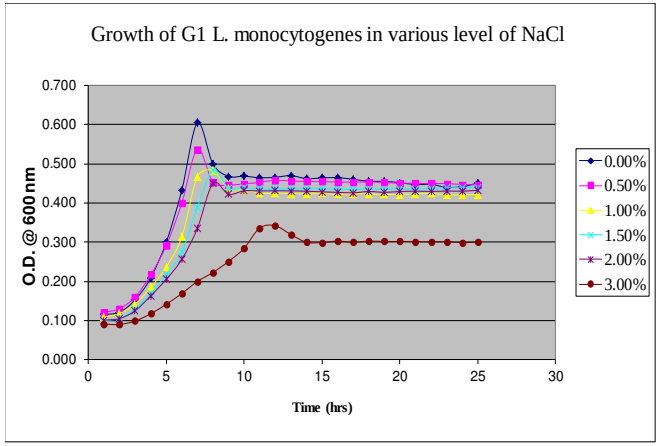
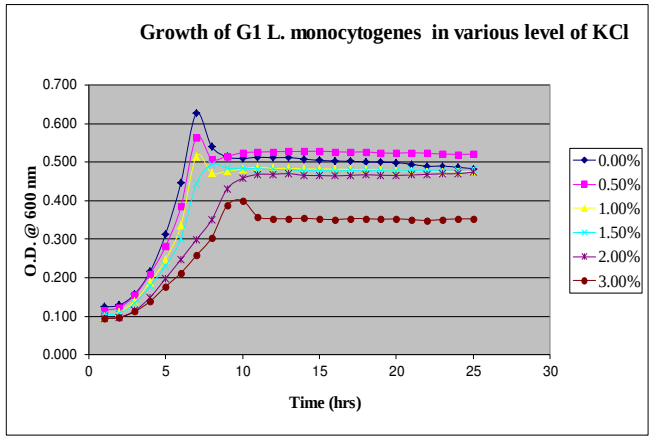


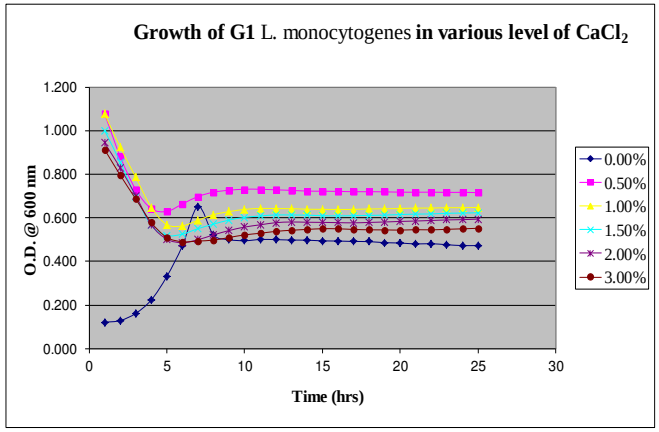
Figure 7.9 L. monocytogenes G1 (A) and G2 (B) growth curve in Milk Plate Count (MPC) Broth, unsalted liquid phase of spread and unsalted liquid phase of spread with potassium sorbate (0.063%)



A

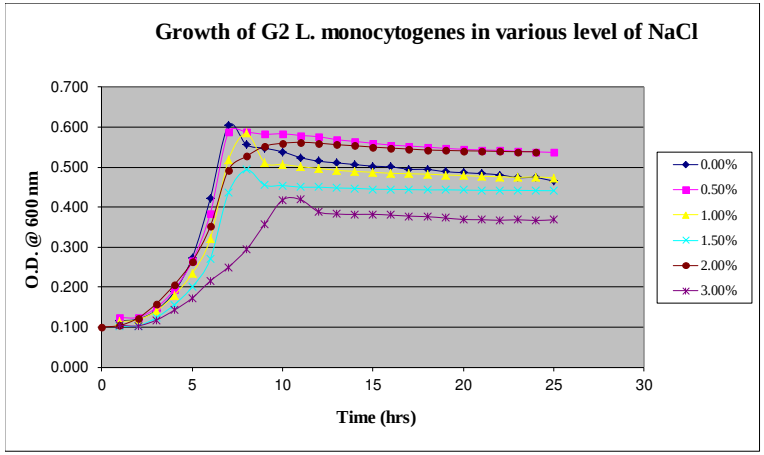


B

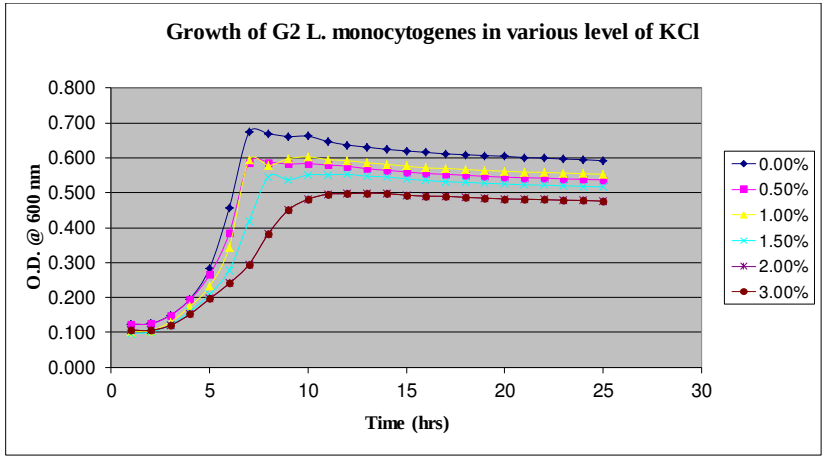


C

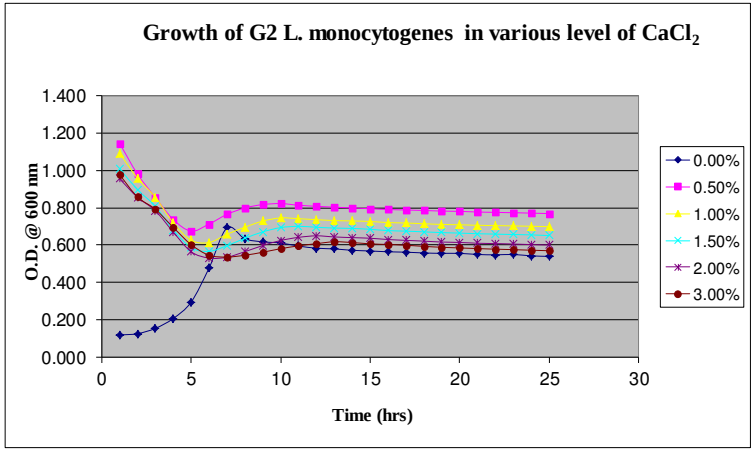
Figure 7.10 L. monocytogenes strain G1 growth in Milk Plate Count (MPC) broth with various levels of NaCl (A), KCl (B) and $CaCl_2$ (C)



A



B



C

Figure 7.11 L. monocytogenes strain G2 growth in Milk Plate Count (MPC) broth with various levels of NaCl (A), KCl (B) and $CaCl_2$ (C)

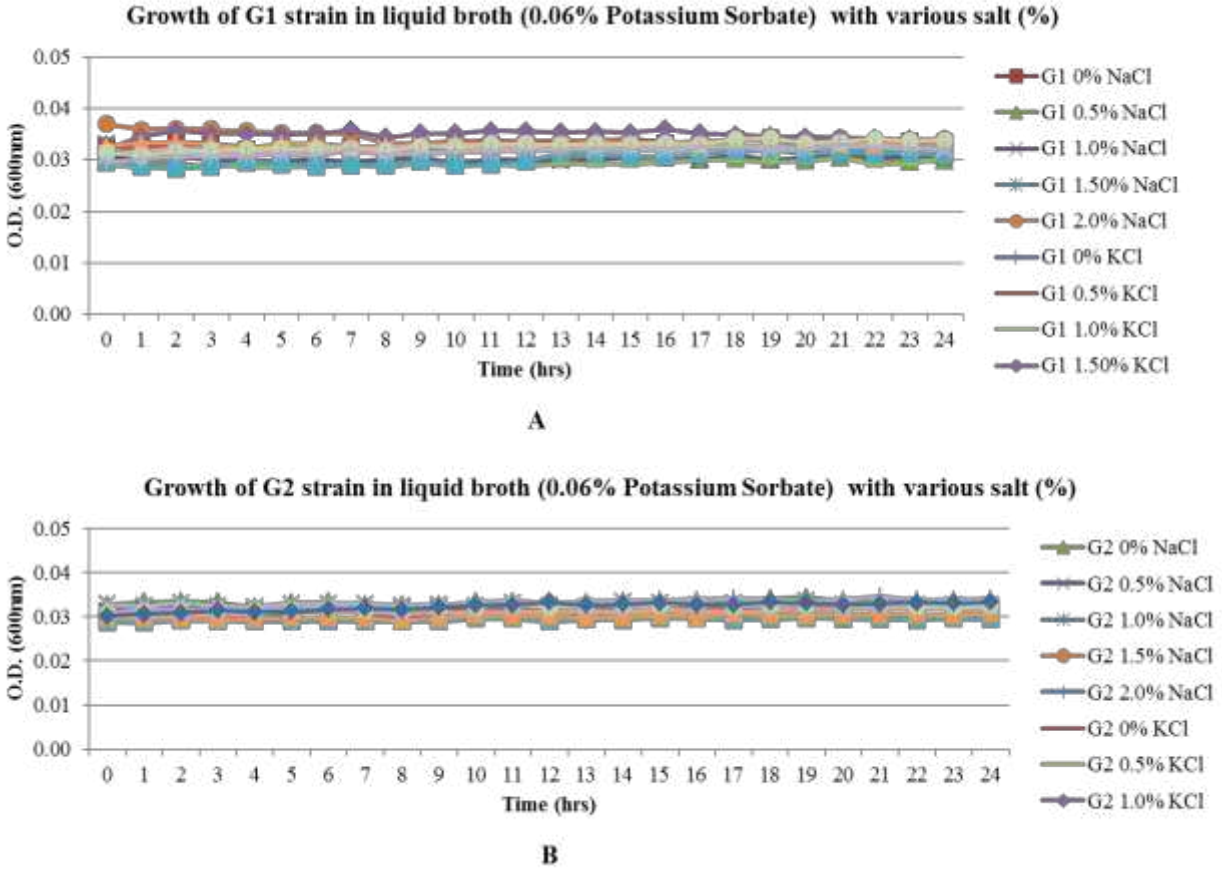
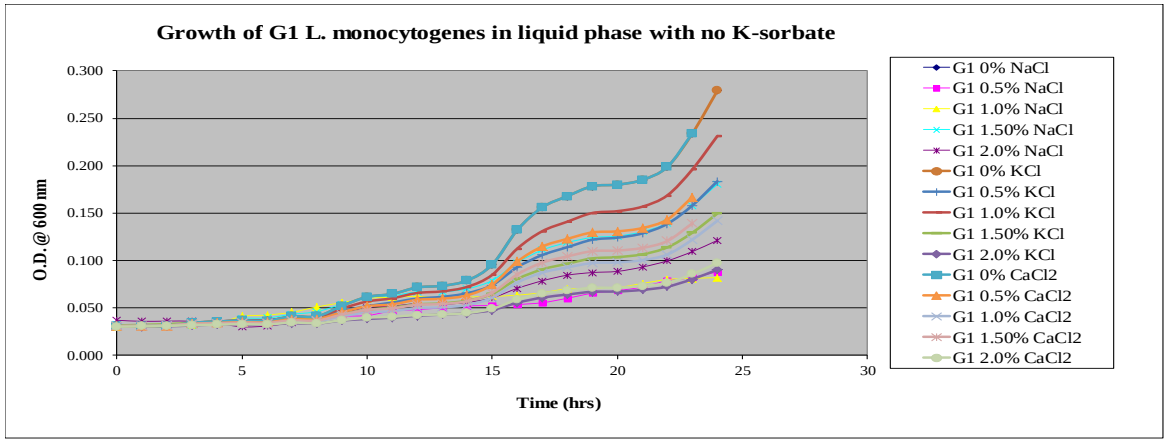
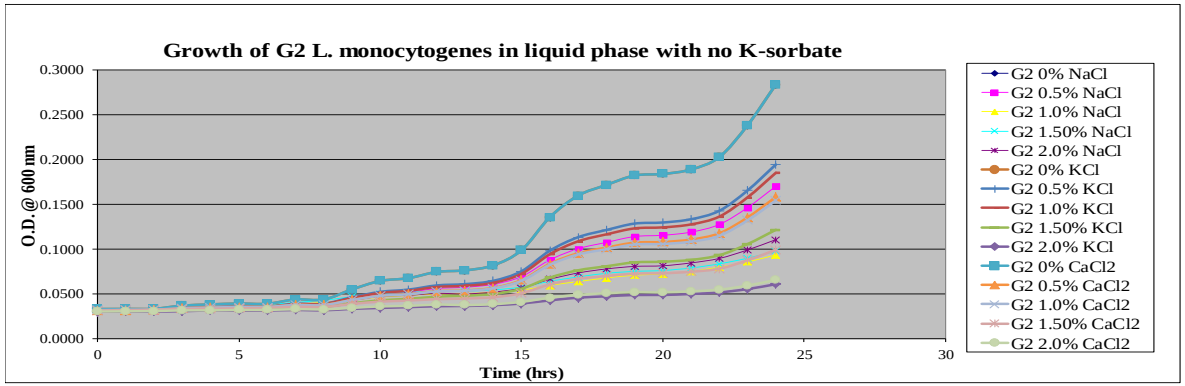


Figure 7.12 L. monocytogenes strains G1 (A) and G2 (B) growth in liquid phase formulation with various levels of NaCl, KCl and $CaCl_2$ with 0.06% potassium sorbate



A



B

Figure 7.13 L. monocytogenes strains G1 (A) and G2 (B) growth in liquid phase formulation with various levels of NaCl, KCl and $CaCl_2$ with no potassium sorbate

7.4 DISCUSSION

Microbial challenge-test aims to provide information on the behavior of microorganisms that have been artificially inoculated into a food under given storage conditions. They take into account the variability of production batches (using different batches) and the specific contamination (inoculating various strains isolated from the food). However, the level and heterogeneity of the contamination and physiological state of the bacteria in a natural food environment and matrix are difficult to mimic in a study. Challenge tests can be performed with two different objectives either to assess the growth potential, or to estimate the growth parameters (e.g. maximum growth rate, generation of bi-products). Microbiological challenge tests play an important role in the validation of processes that are intended to deliver some degree of lethality against a target organism or group of target organisms.

An appropriately designed test will validate that a specific process is in compliance with the predetermined performance standard. A number of factors are associated with a microbial challenge test as (EU Micro Criteria 2073:2005): the selection of appropriate pathogens or surrogates, level of challenge inoculum, inoculum preparation and method of inoculation, duration of the study, formulation factors and storage conditions, and sample analyses.

Listeria spp occur mainly in soil, decaying matter, silage and water. The specific potential sources of food poisoning bacteria in dairy products are from the raw

ingredients, poor hygiene and sanitation during processing, and incorrect processing conditions. Other hazards that are common to all types of food processing (including contamination of foods by insects, glass etc.) are prevented by correct quality assurance, including the design and operation of the processing facilities, staff training in hygiene and production methods, and correct cleaning and maintenance procedures.

The main six strains in the Listeria genus are L. innocua, L. ivanovii, L. welshimeri, L. seeligeri, L. grayi, L. monocytogenes, prevalent in food and its environment. A further four new species have been isolated recently L. fleischmannii, L. marthii, L. rocourtiae and L. weihenstephanensis. Of these species L. monocytogenes is the only one that is pathogenic to humans

Environmental sampling was used to detect the presence of pathogenic and general environmental bacteria in a processing area, prompting their control in the environment before they become a hazard in the food itself. The major routes of environmental contamination can be broadly classified as surfaces, water and air.

Maximum Growth Potential (δ) was >0.5 log for L. monocytogenes in cream, therefore classifying the product as a "Ready-to-eat food able to support the growth of Listeria monocytogenes other than those intended for infants and for special medical purposes" (EU Micro Criteria). On the other hand the spread produced under the SSHE process showed no growth within the product over the

total shelf life showing that finer droplet size prevents bacterial growth even at neutral pH conditions.

Listeria spp is a known pathogen to the dairy industry. The organism has the ability to adhere to surfaces of equipment, filters, floors, mixers, cleaning utensils, stainless steel by forming a biofilm in a more resistance physical state (Holah et. al.,, 2014). The biofilm can be monolayer to few micron thick and cells becoming more resistant to disinfectant being in a sessile state. Hence the dairy isolates were further challenge tested at the pasteurisation and CIP cycle time and temperature combinations and no survival was obtained stating the isolates are not a resistant strain within the dairy. It could be further concluded that the contamination was more likely to be post process rather than survival through the plant itself. The cream is produced as a bi-product of the milk manufacturing site that is then further processed into spread and butter.

While challenge testing the organisms at various emulsion formulations (salt and pH), the results showed similar pattern to the earlier microbial challenge test where low pH (≤ 5.5) showed slight reduction to the growth rate. Due to the product standard flavour profile the pH alteration was not an option and with the salt level being regulated by FSA on the level of sodium present in the product, the test was further expanded to understand the antimicrobial activity of potassium and calcium salt as a replacement to sodium. The water phase of the spread formulation was used for this study as the organism will grow within the

available water within the water droplet size. No increased hurdle rate was observed in other salts as KCl or $CaCl_2$ compared against NaCl.

One of the constraint on using various salt were with $CaCl_2$ giving a different growth curve to the other with a high initial optical density that decreased over the first 5-7 hours may be as a consequence of the formation of an insoluble calcium sulphate salt. The composition of the broth contains sulphate ions that would react with the calcium chloride to produce calcium sulphate which in the course of time would settle out. After the salt settlement stage at 10 hrs, the growth curve was very similar to that for the other two salts hence $CaCl_2$ did not contribute any extra hurdle rate to the organism growth rate when compared with NaCl and KCl that is currently used in the industry.

CHAPTER 8 OVERALL DISCUSSION

Spreads exist as an emulsion during the production process (Dickinson 1989; Friberg and Yang 1998, McKenna 2003). The overall qualities of an emulsion-based product are dependent on a combination of physiochemical and sensory characteristics and also exhibit a great diversity of rheological characteristics in viscosity. The raw materials such as the water, oil, emulsifiers, thickening agent, minerals, acids, vitamins, flavour and processing conditions (mixing, homogenization, pasteurisation etc.) determines specific quality attributes of a product. Emulsion is pasteurised prior to filling into the finished packaging and stored and distributed under refrigeration conditions with a shelf life ranging from 10 to 12 weeks in a standard spread. Spread is suitable for spreading, cooking, frying and freezing.

Emulsion breakdown is influenced by environmental factors such as pH, temperature and processing conditions (Rousseau 1999). They lower the interfacial tension between the oil and water phases and also form mechanically cohesive droplets thus preventing coalescence, water acts as a continuous phase and oil as the dispersed phase and the particles embed within the interface at an equilibrium distance. To form a stabilised O/W emulsion the contact angles of the particle/ oil/ water interface has to be smaller than 90º; if greater than that the particle will stabilise a W/O emulsion (Rousseau 1999). The process that involves converting the two immiscible liquids into an emulsion is called homogenisation carried out mechanically by a homogeniser. This is usually

carried out in two steps, primary and the secondary homogenisation. In the former, involves formation of an emulsion primarily from two separate liquids, whereas the latter will reduce the size of water droplets in the existing emulsion (McClements 1999) and gives rise to smaller droplet size.

The preservation system depends on the presence of a fat-continuous matrix; any water present is in the form of very finely dispersed droplets. Due to this dispersion, microbial contaminants are restricted in growth either by space limitations or by exhaustion of the nutrients in the droplets. The inability of microorganisms to move between water droplets is a major intrinsic preservation factor (ICMSF, 2005). Fat can act as a barrier to microbial growth, resulting in much more stable fat continuous systems than water continuous systems. This generally limits microbial growth, but does not necessarily exclude growth under extreme conditions (ICMSF, 2005). The ability of a microorganism to grow in an emulsion depends partly on the volume of the water droplet it finds itself in (Charteris 1995).

Emulsion stability is dependent on the uniform distribution and the interfacial tension between the two phases. The process for emulsion breakdown (Figure 8.1) is aggregation (flocculation), coalescence and phase separation (Figure 8.2). Flocculation is a process where emulsion drops aggregates without rupture of the stabilizing layer at the interface. It may occur under conditions when the van der Waals attractive energy exceeds the repulsive energy and can be weak or strong, depending on the strength of inter-drop forces. (Rajah 2002). Coalescence is an

irreversible process in which two or more emulsion drops fuse together to form a single larger drop where the interface is ruptured. The interaction of the two drops across the film leads to the appearance of an additional disjoining pressure inside the film (Figure 8.3). The phase separation is a process of flocculation and coalescence are followed by phase separation, i.e. emulsion breakdown.

The aim of this research was to develop a risk assessment tool to produce a food safe and quality product (Figure 8.4) based on the complaint and spoilage issues on Spread A in 2009. The company produces various different types of spread with different formulations and two different processing conditions as churn and SSHE. All the complaint were on Spread A and A lighter, which were being produced at factory A in one production site under the traditional churn method. Whereas spreads made in factory B under the SSHE did not show any spoilage issue even though the intrinsic parameters as pH, aW and salt % were similar.

One of the routes of product breakdown is via microbial contamination (Aprigny and Jaeger 1999). Certain groups of microorganisms can be characterized by the way they metabolize the product fat and break it down (Allen and Hamilton 1994; McKenna 2003). The main possible organisms that may cause lipolytic microbial spoilage or product quality issues are Pseudomonas spp causes black, green or metallic discolouration of the product followed by fruity or metallic taints (Allen and Hamilton 1994). Bacillus spp may cause black discolouration and result in ropiness as a sign of spoilage as the spores start germinating at 4ºC (Allen and Hamilton 1994; McKenna 2003). Presence of Micrococcus spp can produce heat

stable lipases and moulds show visible growth in the product and they contain reactive lipases and free fatty acid (FFA) oxidative enzymes. Yeasts are usually salt and acid tolerant and hence are one of the main organisms that causes quality issues (Allen and Hamilton 1994; McKenna 2003).

As spreads become rancid (undergo breakdown of the fat), components are produced which may give rise to changes in both physical and organoleptic properties of the product. In general the liberated free fatty acids and peroxides thereof are of most interest. However the fatty acids can be broken down still further into smaller volatile molecules such as aldehydes and (methyl) ketones (Stead 1986; Allen and Hamilton 1994; Brocklehurst 1995). Many of the fat breakdown products have characteristic odours and can give rise to unpleasant taints within the product making it undesirable to the consumer.

The mean diameter of the monodispersed droplet size in the aqueous phase of a spread is usually between 4 & 5 μm, but can range between 1 and 30 μm (Charteris 1995). Microorganisms cannot grow in emulsions with a droplet size of less than approximately 10μm (Boysen 1927). The distribution for Y&M is considered to be similar (Charteris 1995). But coalescence of droplets can release water at the product surface allowing mould

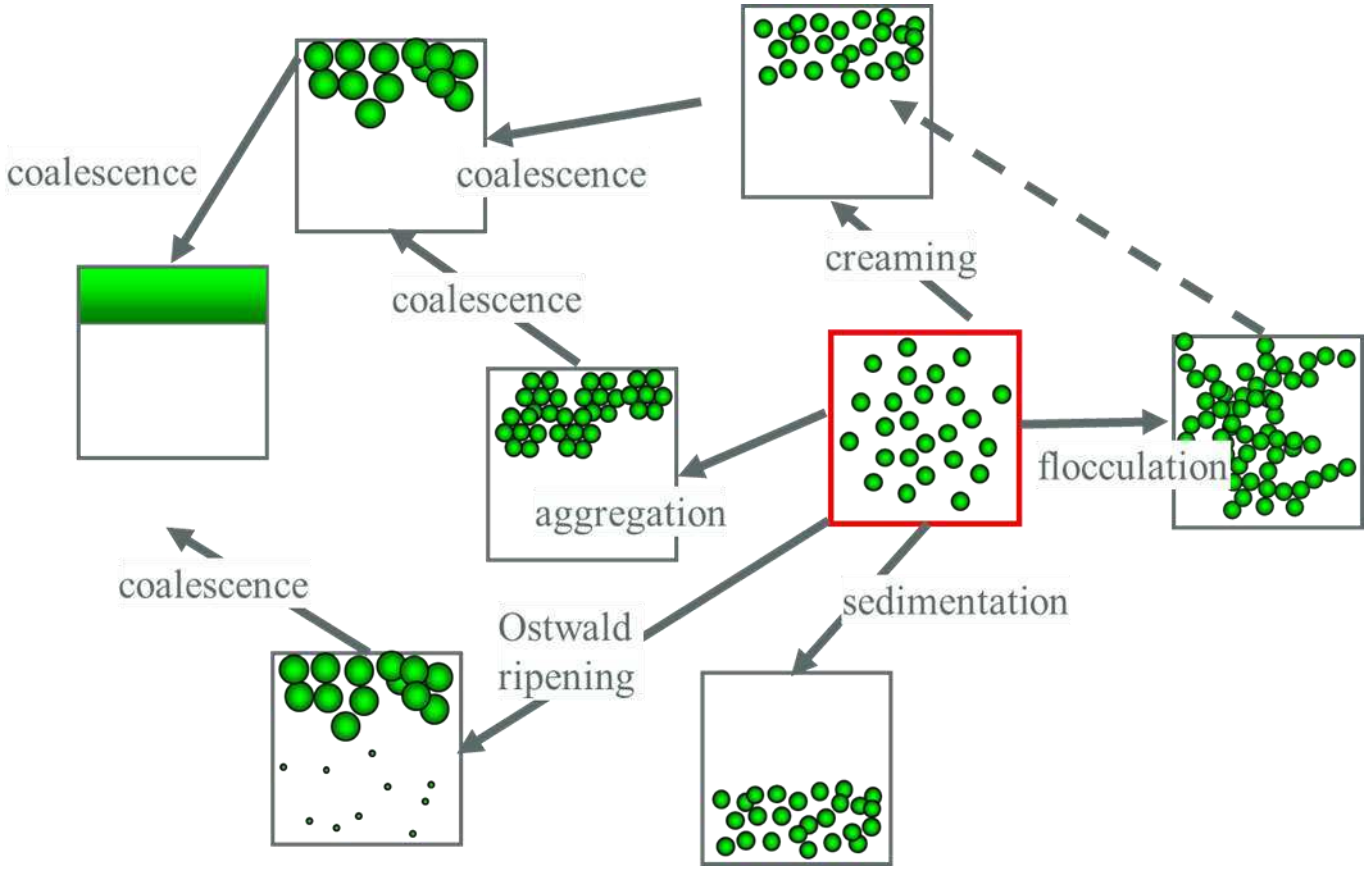


Figure 8.1 Emulsion instability process

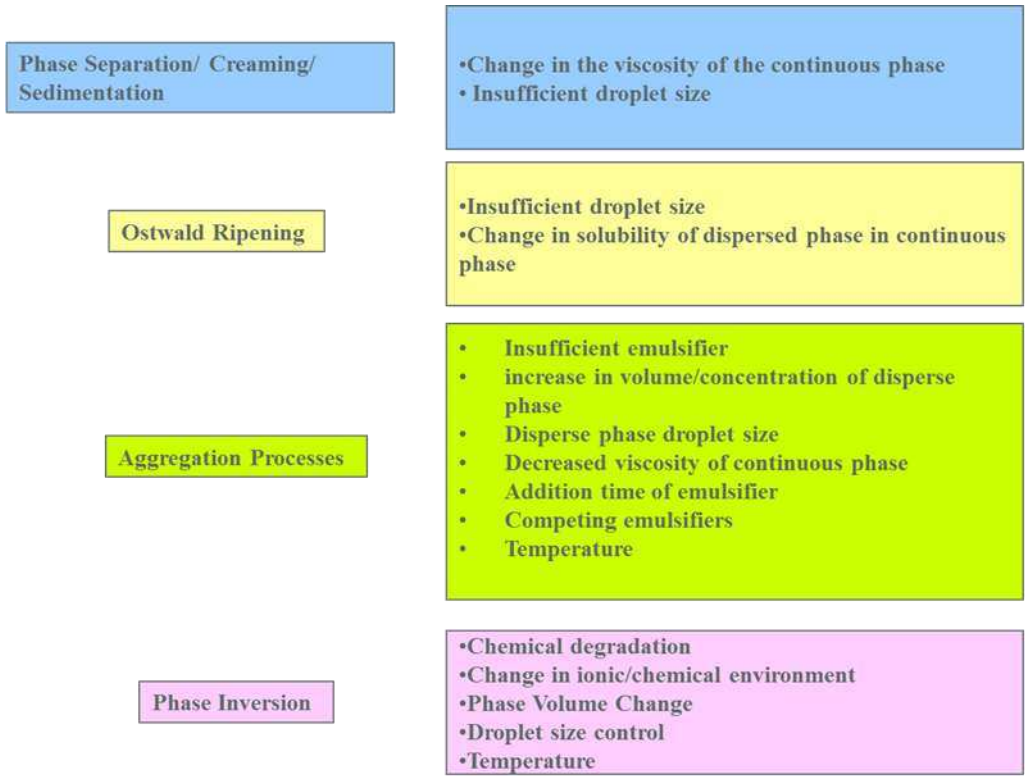
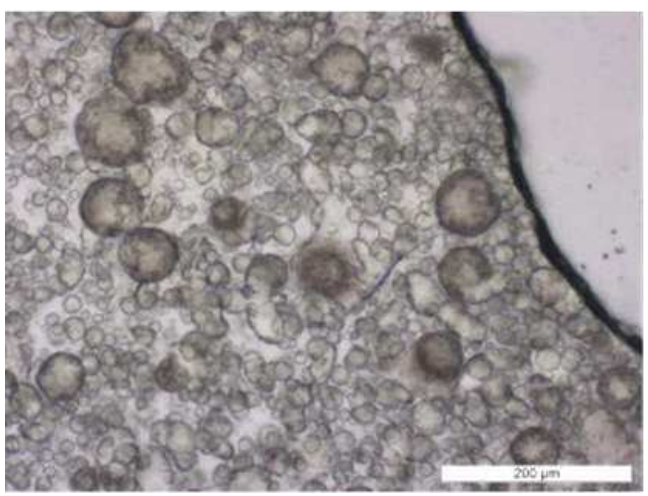
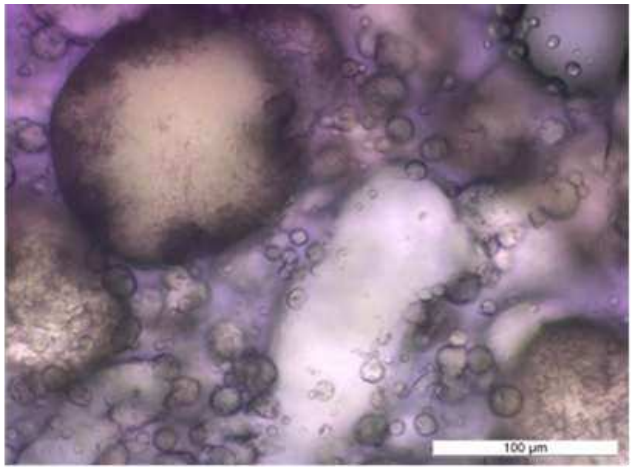


Figure 8.2 Emulsion instability stages



Stable Emulsion



Phase Inversion

Figure 8.3 Phase inversion of emulsion

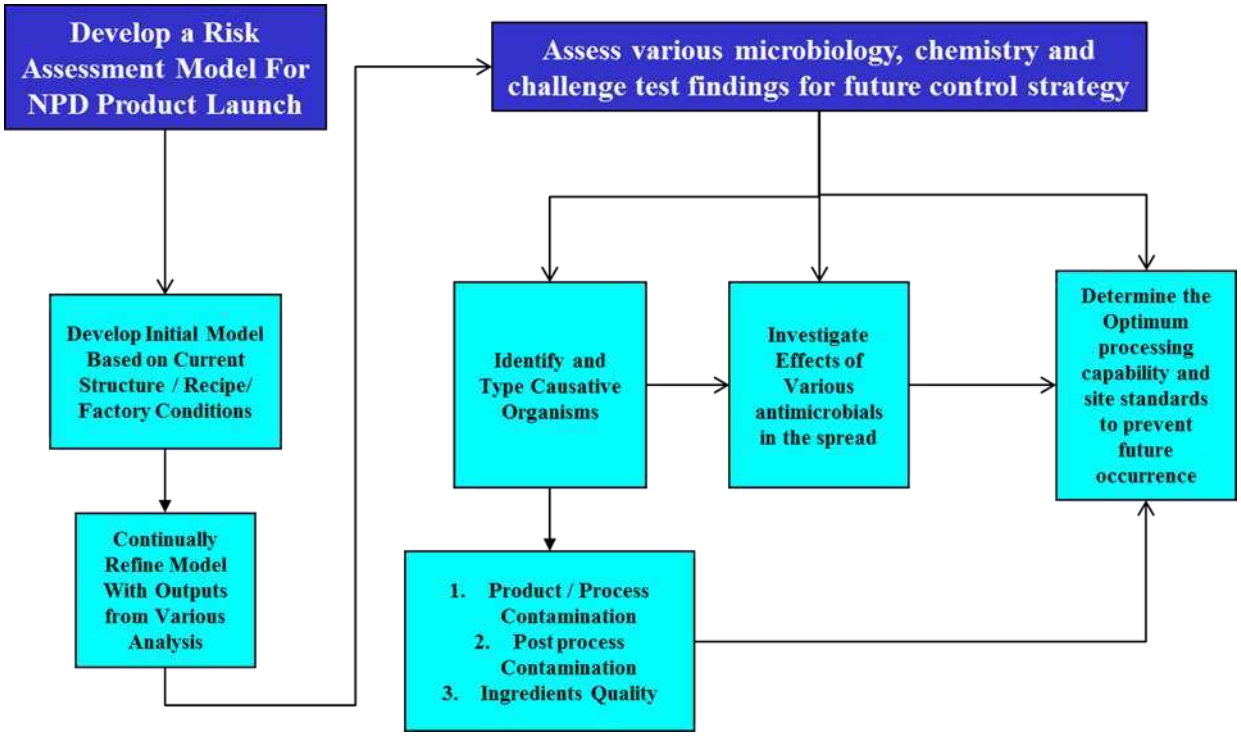


Figure 8.4 Overall risk assessment layout to prevent future spoilage issues based on the findings in this study

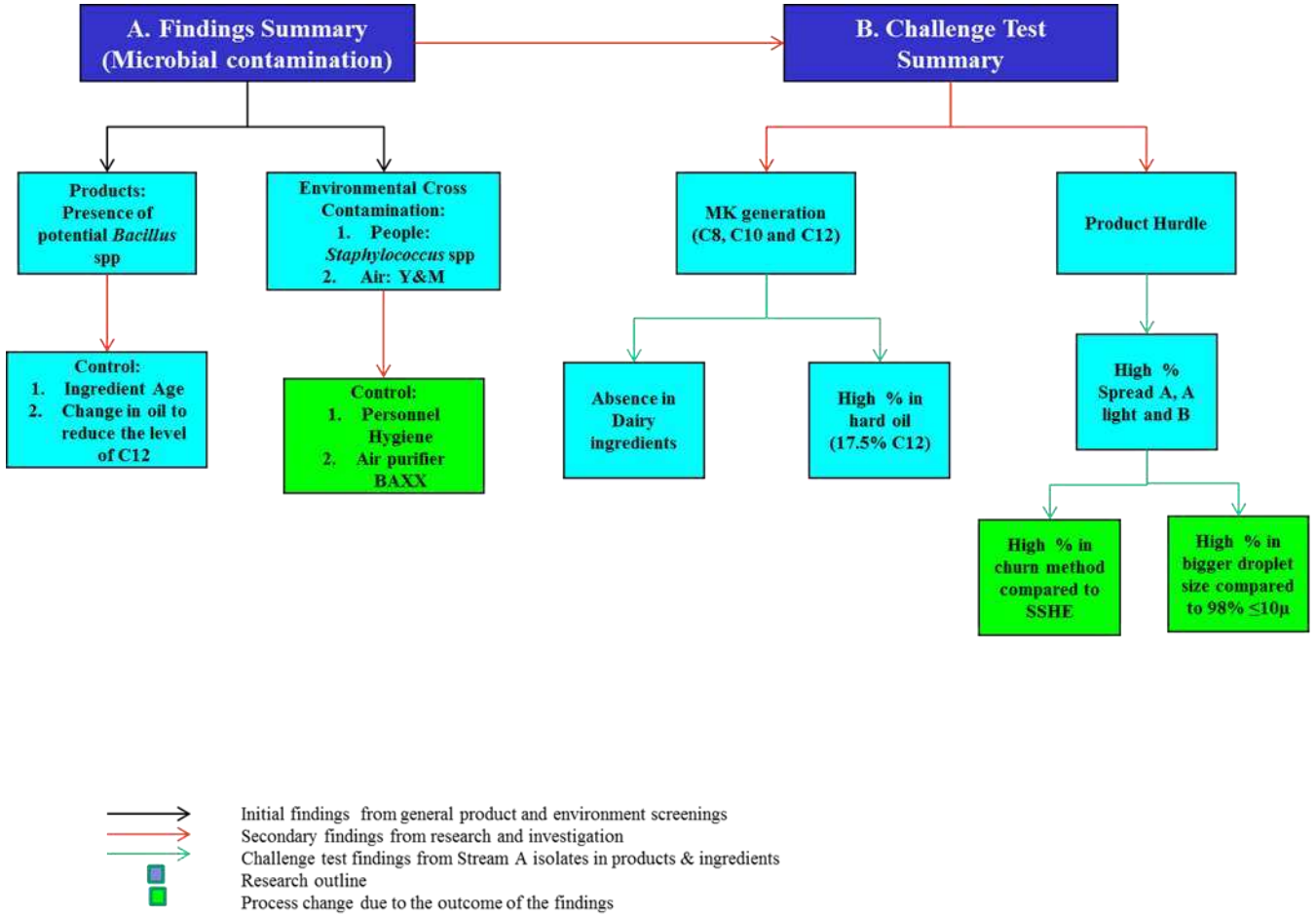


Figure 8.58.4 Summary of overall research findings and further processing

One of the routes of product breakdown is via microbial contamination (Aprigny and Jaeger 1999). Certain groups of microorganisms can be characterized by the way they metabolize the product fat and break it down (Allen and Hamilton 1994; McKenna 2003). The main possible organisms that may cause lipolytic microbial spoilage or product quality issues are Pseudomonas spp causes black, green or metallic discolouration of the product followed by fruity or metallic taints (Allen and Hamilton 1994). Bacillus spp may cause black discolouration and result in ropiness as a sign of spoilage as the spores start germinating at 4ºC (Allen and Hamilton 1994; McKenna 2003). Presence of Micrococcus spp can produce heat stable lipases and moulds show visible growth in the product and they contain reactive lipases and free fatty acid (FFA) oxidative enzymes. Yeasts are usually salt and acid tolerant and hence are one of the main organisms that causes quality issues (Allen and Hamilton 1994; McKenna 2003).

As spreads become rancid (undergo breakdown of the fat), components are produced which may give rise to changes in both physical and organoleptic properties of the product. In general the liberated free fatty acids and peroxides thereof are of most interest. However the fatty acids can be broken down still further into smaller volatile molecules such as aldehydes and (methyl) ketones (Stead 1986; Allen and Hamilton 1994; Brocklehurst 1995). Many of the fat breakdown products have characteristic odours and can give rise to unpleasant taints within the product making it undesirable to the consumer.

The measurement of specific breakdown products that liberate methyl ketones can be a useful tool in predicting the nature of the microbiological contamination. Four key microbe groups that produce high levels of methyl ketones are Bacillus spp, Staphylococcus spp and yeasts and mould (Allen and Hamilton 1994; McKenna 2003). As the ingredients in the spread production are heat treated (pasteurised usually >80ºC due to high fat content), it is highly unlikely for any microbes to be present unless they are bacterial spores that can survive the heat treatment (Rarier and Boor 2009) and can also survive in the storage tank for a long time. The product can be contaminated from ingredients, post process by the packaging or the factory environment thus reintroducing less heat resistant organisms in the process.

From the study it was noted that buttermilk, sweet cream buttermilk and skimmed milk contained a high percent of Bacillus spp (66%), Staphylococcus spp (60%), yeasts (>70%) and few moulds. After screening the spoilage complaint products along with freshly produced samples it was identified that the main contaminants were microbial. The bacterial strains identified by 16S rDNA sequencing were Staphylococcus equorum, Staphylococcus pasteuri, Staphylococcus sciuri, Bacillus subtilis and Bacillus licheniformis and Pichia guilliermondii, Candida guilliermondii, Candida parapsilosis and Candida ortho parapsilosis as the Y&M strains..

The water was of the lowest risk with practically no microbes present. The oil was of good microbiology quality with only 10% of samples positive with

Bacillus spp and all the counts were less than 103 cfu/ g as required by the product specification aligned to the EU Micro Criteria (2075/2003) and PHE guideline (2009). The product silo showed a higher 'per-cent' of presence of the causative microbes comparing to that of the filler. The silo holds 120,000l of product over the 72-hour run-period before being cleaned (CIP). Similar to the products, the age of the ingredient were also noted while analysing for the presence of microbes. It was observed that if any of the high-risk ingredients (sweet cream buttermilk and skimmed milk) were more than 42-hours old from the last pasteurisation, the microbial load significantly increased at least by one to two fold. As per 'Dairy products hygiene regulation' (1995) the age of raw milk should be less than 48 (ideally 24 h) prior to further processing as this prevents heavy contamination in the milk from the natural flora of the udder and milking parlour. It is known that pasteurisation can only give five to six log reduction of bacterial growth, hence if the raw material is heavily contaminated, the contaminant will be carried through to the finished product and affect the quality or safety of food (Andrew 2003). Based on these findings as part of GMP, the processed cream &and buttermilk age for spread making was restricted to 24 h prior to use.

To identify the causative microbes, 50 isolates from both product and environment were analysed giving a satisfactory result of sequence matches of greater than 97% base pair matching in all cases. The strains identified by 16S rDNA sequencing were Staphylococcus equorum, Staphylococcus pasteuri, Staphylococcus sciuri, Bacillus subtilis and Bacillus licheniformis. All these

strains matched with the identification with the API testing other than S. pasteuri. In the API STAPH S warneri was identified. S pasteuri could be identified as S. warneri in the API STAPH system as they are phenotypically similar but genetically more closely related to each other (Chesneau 1993).

Pichia guilliermondii, Candida guilliermondii, Candida parapsilosis and Candida ortho parapsilosis identified with more than 98% base pair matching. The moulds identified were Penicillium brevicompactum and Penicillium crocicola with more than 97% base pair similarity. P. brevicompactum is xerophilic in nature and can spoil refrigerated products such as cheese, margarine and dairy products (Pitt and Hocking 2009). All the isolated strains from the 'cheese flavour' samples (Bacillus spp, Staphylococcus spp, yeasts and moulds) showed lipolytic activity. Lipase enzyme hydrolyses glycerides to form free fatty acids; in butter and other dairy products this can cause rancidity (Allen and Hamilton 1994 and McKenna 2003). The characteristic odours of rancid dairy products are caused by the release of butyric acid - a volatile fatty acid. Nearly, 80% of the skimmed milk and sweet cream buttermilk (pre-pasteurisation) were positive for lipolytic microorganisms. The percentage of lipolytics nearly halved in the sweet cream buttermilk after pasteurisation, that may indicate as the main contaminant being Bacillus spp, which are spore former and can survive pasteurisation temperature. The cream and buttermilk also had higher number of lipolytics, but the total percentage was a lot lower, 14 and 19% respectively. The oil and water were of lowest risk, as it would be assumed due to the nature of the product. The ingredients that showed the presence of the causatives spoilage organisms are all a

bi-product of the site from processing milk and cream hence by limiting the age of them prior to further processing will lower the level of initial contaminant. The source of the Staphylococcus spp and Y&M were post pasteurisation contamination from personnel and factory air. The Staphylococcus spp were controlled from proper GMP training in the hand wash area and handling of the post pasteurised products. An air purifier system BAXX was installed around the factory and the air was sampled for a further 15 months after the installation of the two BAXX units across the production hall near the filling machines. It has shown significant reductions of the contaminants and improving the general environment microbial load (TVC ≤ 100 cfu/l) and for Y&M a 100% pass rate was observed.

One of the key spoilage indicator in the products was the development of the distinct 'cheese flavour' due to the breakdown of fats to further aldehydes and ketones. It was observed that more than 50% of the samples contained free fatty acid (FFA) C8, C10 and C12 that give the 'cheese flavour'. 62% contained C10, 52% C12 and 32% had C8, compared to only 7% of C6. Also, octanoic, decanoic, dodecanoic, tetradecanoic (C14) and hexadecanoic (C16) were present showing breakdown of triglycerides. Product rheology and free fatty acid analysis (methyl ketones) of the standard products showed relatively as a whole higher levels of C12 compared to the ones in the market. C12 on its own in the product does not exert 'cheese flavour' smell unless it is further utilised by microorganisms or contaminants. On the other hand, amongst the other product ingredients the oils showed a higher percentage of C12 (similar to those of the 'cheese flavour'

samples). Hence relatively low risk product oil containing high level of C12 can generate spoilage once organisms are introduced in the formulation through high risk ingredient cream and buttermilk.

The factory runs continuously for 72 hours, after which a deep clean is performed and the next batch starts on the following morning at 6 AM. While sampling the air as a part of a routine monitoring programmer it was observed that there were high levels of yeast and moulds on day one and the level slowly dropped off in the three-day period. When analysing the spread samples it was also observed that the contamination level was higher on the first 24-hour production run compared to the samples on the latter run time. If the source of cross contamination were cleaning, processing or pasteuriser run time issue, we would expect the level to be greater on the third day of the run instead of being on day one. This indicated that the source of contamination is more likely to be the environment. Therefore the environment of the factory was screened for the presence of spoilage organisms. The predominant floras in the environment plates were a variety of yeasts and moulds that looked morphologically similar to those of the product and ingredients. All the other microorganisms such as Enterobacteriaceae, E.coli, Salmonella spp, Bacillus spp, Staphylococcus spp, Micrococcus spp, Listeria spp, Pseudomonas spp, proteolytic and lactic acid bacteria were absent. All the strains of yeast and mould also showed similar level of lipolytic activity as those of the product and ingredients.

The factory air is filtered and the pressure is monitored. But the spread site has a moist filling area and it is not filled in aseptic filler. Therefore the product may get contaminated after processing. Moreover there is only a coverleaf on the product, not foil like all other competitor products. It has also been noted that the products contaminated with microbes are relatively softer in texture at 20ºC comparing to the standard product. This also denotes that the product is being broken down by the contaminants.

An air purifier system BAXX was installed around the factory after the findings. The Baxx system uses novel cold plasma technology to produce a highly active antimicrobial environment, which kills all known airborne and surface-active pathogens including, viruses, moulds, bacteria and spores (BAXX user manual 2010). BAXX proprietary patented process works on a discharge based non-thermal plasma ion multiplier. A burst of high energy electron (1`10eV) is created in an ambient environment consisting of air, water moisture and pollutant where the electrons collide with the dominant background molecules, creating highly active radicals reacting against bacteria, virus and Y&M without the use of toxic compounds within a food factory. The unit has shown an effectively kill on Escherichia coli, Staphylococcus aureus, Campylobacter spp, Listeria monocytogenes, Pseudomonas spp and general Y&M populations, this was validated by the BAXX providers by performing a challenge test at Campden (data not presented). The factory air was sampled for a further 15 months after the installation of the two BAXX units across the production hall near the filling machines. It has shown significant reductions of the contaminants and improving

the general environment microbial load (TVC ≤ 100 cfu/l) and for Y&M a 100% pass rate was observed.

As per Figure 8.5, the isolated strains were used as a library strains further in the study to determine the process optimisation conditions and capability depending on the product hurdle factors. The dairy derivatives as cream and butter milk were high risk ingredient but the presence of high levels of C12 in the hard oil blend of spread A and A lighter made it more prone to spoilage with the presence of the causative organisms.

The standard spreads while being challenge tested by the causative organisms, it was observed that the product requires the presence of Bacillus spp or Y&M to breakdown the product oils or fats to release different FFA that later on exerts 'cheese flavour'. The hard oil blend seemed to be the main source of methyl ketone generation. Air purifier BAXX also helped to reduce air borne cross contamination with yeast and moulds. This in the long term also helped with generation of little FFA in the product, one of the main biochemical products that is responsible for the distinctive 'cheese flavour'.

The overall aim of this study was to understand and eliminate the root cause of the 'cheese flavour' spoilage problem in the spread. One of the main things was to determine the product microbiology product hurdle rate in respect to the percentage of moisture and salt in moisture. The droplet size is one of the limiting factors to microbiological growth (McKenna 2003). The product firmness is also

dependent on the droplet size. The spread texture when compared with the 'cheese flavour' product against a standard product, it was noted that the top layer was significantly softer in the former. The mean firmness value for the top layer distribution of the samples were 10% lower in the 'cheese flavour' with subsequent layers being much the same across all samples as the middle and bottom layer rheology was consistent with the variability standard deviation being ~ 14%.

The hurdle rate in a product ensures that the food has a controlled environment to either eliminate or reduce the growth of pathogens to prevent it to become harmful to humans (Mckenna 2003). Hence it renders it safe for consumption and also helps to extend the shelf life of the product. The right combination of hurdles can ensure all pathogens are eliminated or rendered harmless in the final product. Some examples of hurdles in food are high temperature processing, low temperature for storage, acidity, water activity, redox potential, salt or the presence of any other preservatives (Jay 1996). According to the type of pathogens and its pathogenicity the intensity of the hurdles can be adjusted to meet product quality and consumer preferences. The main organisms concerned in this study were Listeria spp, Bacillus spp and yeasts &and moulds, as these organisms were mainly associated with the spreads spoilage.

The pH of the aqueous phase is also a hurdle factor for microbial growth as is droplet size. The pH is usually controlled by the addition of an organic acid such as lactic acid. Most bacteria are inhibited by acidic, low pH conditions and

Listeria monocytogenes in particular will not grow at pH levels below 4.5 (Voysey 2000). Unsalted and slightly salted products generally contain lactic acid for flavour and to make up for the reduction in hurdle rate from the removal of salt and this is reflected in the low pH of the competitor products. The near neutral pH levels of Spread A, A lighter, B & C are associated with salted products, however, at these sort of pH's bacterial inhibition is minimal as observed in various growth curves in pH ranging from 5.0 to 6.5. The disadvantage of reducing pH within the spread A formulation process is that there is a significant change in flavour; flavour release and mouth feel i.e. would no longer taste like the standard product.

The level of salt dissolved within the aqueous phase of the product (% salt in moisture) is a further hurdle that controls microbial growth (Mckenna 2003). C and B spreads were the two spreads with the highest salt in moisture levels which reflects the need for this hurdle to compensate for the reduction in hurdle rate afforded by pH. Of the competitor samples analysed most either met or approached closely the 2012 FSA salt target of 1.15% salt but they all had acidic pH compared to the neutral pH of spread A. On the other hand spread A, A light, B, C, C light & D contained salt levels above 1.5% with or without the additive potassium sorbate. Hence salt level couldn't be raised any further to improve the hurdle rate of the product.

On top of all the factors stated above as the salt &and pH are the largest hurdle within yellow fat spreads technology is along with the size of the moisture water

droplets and their uniform distribution. distributed within the product as mentioned above. To sustain microbial growth the accepted literature value for minimum droplet size is >5 μ in radi (McKenna 2003). In the comparison study of the moisture droplet distribution in standard spread formulation (A, A Lighter, B, C, C Lighter & D) and competitor spreads (E, F Butter, F Salted, F Unsalted, F Light, G & G Light) it was noted that 'A' and 'B' had a poorer droplet size distribution 95% ≤ 10 μ (Figure 5.3) with former being significantly lower than the other market competitors around 68%, whereas 'C' and 'C lighter' achieved 98%≤5 μ level. The majority of the competitors (F Butter, F Salted, F Unsalted, F Light, G & G Light) were achieving 98% ≤ 5 μ other than spread 'E' at the level of 90% ≤ 5 μ thus leading to an inherently more microbial stable product.

This difference in droplet distribution may be linked to the process technology utilised to produce the product and specifically the level of secondary homogenisation the product gets from additional mixers. In the case of spread 'C' and 'C lighter' the droplet distribution is good (greater than 98% ≤ 5 μ) as significant levels of secondary homogenisation are part of the production process (McClements 1999). The SSHE process produces fine droplet size, that This in turn leads to a greater degree of microbial stability and hence more robustness against 'cheese flavour'' causative organisms, along with imparting . It should be noted that droplet size distribution plays a part in product texture and mouth feel. Having too narrow droplet size distribution i.e. high levels of small droplet sizes can lead to a greasy mouth feel that reduces consumer acceptance.

All microbes require water for growth, the measure for how much water is physically available for microbial growth is the water activity (aW) of the product. The water activity of a product is principally affected by the amount of water within the product and the concentration of solutes such as salt dissolved within that water. Traditionally aW is the principal measure utilised in growth prediction software, however, aW needs to be considered in the context of the product matrix under review. Within yellow fat spread aW gives an overall indication of the susceptibility of the product to microbial growth this cannot be considered as an exact comparison due to chemical interactions that occur at the microscopic level. Solutes dissolved within a water droplet will not be uniformly distributed within that droplet. Due to interactions at the lipid/water phase interface significant pH and salt gradients occur throughout the droplet and this results in regions of low and high aw within the droplet. Currently these interactions and gradient effects have not been fully researched by the academic world so aw comparisons can only be made on a macroscopic level. Having examined all these aspects it was decided that the solution to the problem would be a methodology that would increase the product hurdle rate. The scrape surface heat exchangers would produce better and finer water droplet size, which on the other hand creates a good microbiological hurdle to product spoilage.

Spread A and A lighter after being produced by the scrape surface heat exchanger instead of the traditional pasteurisation churn process, a finer droplet size distribution to a minimum of 90% $\leq$ 5 μ was achieved. Spread C & C lighter were already produced by the scrape surface heat exchanger method and hence had

better droplet distribution. The trial products were further analysed for methyl ketone generation and showed a 50% reduction in the generation of the ‘cheese flavour’’ methyl ketones, i.e., pentanone and hexanone were observed in 100 new and STD products. On day 7 no MK generation was found for the new formulation product and the control, whereas the standard product showed the presence of breakdown of the product. The same was also observed on the 14-day test. Hence the new products were more robust against the growth of the causative organisms giving a ‘cheese flavour’’ spoilage aroma.

To evaluate further robustness of the product the trial product from the SSHE process when challenge tested L. monocytogenes over 10 week shelf life period, the organism was not able to grow. The liquid phase of the emulsion was further challenge tested with L. monocytogenes isolated from various part of the dairy environment with various salts at different concentrations to understand the antimicrobial effect of sodium, potassium and calcium . salts. No difference in the growth rate was observed. This data can lead to the generation of a growth predictor of the organism at 0, 0.5, 1.5, 2.0% concentration of NaCl, KCl, and CaCl2 with or without the presence of potassium sorbate. It was observed here as discussed before the pH range of 5.5 or lower due to the presence of 0.063% potassium sorbate showed significant antibacterial affect compared to the nutrient enriched MPC-broth and the unsalted liquid phase with no potassium sorbate.

Hence validating finer droplet size restricts microbial growth. Further the organism couldn’t withstand the heat treatment as pasteurisation and the standard

CIP cycle of acid and caustic treatment and no recovery rate was observed. It could be further concluded that the contamination within the industry is more likely to be post process rather than survival through the plant itself as per RASFF alert of Listeria spp outbreak in dairy. Hence reducing the available water in the liquid phase of the spread and will be a limiting factor to the microbial growth.

CHAPTER 9 FUTURE WORK

The future work for this research would have to concentrate on the Bacillus spp contamination as this aspect has not been looked into. Understanding the growth model of Listeria spp in the product and at different salt levels will need further examination.

The presence of Listeria spp is widespread, although in low numbers, in the production environment and in food. The principal root of transmission of Listeria spp is consumption of contaminated food. The organism can survive many weeks in chilled and frozen foods. Microbiological and epidemiological evidence support an association of the organism in different food types such as dairy, meat, vegetable, fish and shellfish, in both sporadic and epidemic listeriosis. Food associated with transmission often shows such common features (McLauchlin, 1996a) the ability to support the multiplication of L. monocytogenes (relatively high water activity and near-neutral pH); relatively heavy contamination (>1000 organisms per g) with the implicated strain; processed, with an extended (refrigerated) shelf life; consumed without further cooking. Almost all of our food types fall in one or more of the above categories. Hence dairy products are more susceptible to the growth of Listeria spp. Different antimicrobial (hurdle) agents can help minimize their growth. Active HACCP and GMP procedures can further prevent the cross-contamination. Salt is one of the most effective antimicrobial agents that affect the water activity, though indirectly, of the product.

Growth at low temperatures is affected by salt concentration (Schaack and Marth, 1988 a,b,c). The maximum concentration of salt (NaCl) allowing growth is about 12% w/w. Interactions between sodium chloride, temperature and pH have been shown, with growth recorded in the presence of 10% NaCl at 25^{o}C, pH 5.0 after 41 hours, pH 6.0 and 7.0 after 31 hours and pH 8.0 after 35 hours (McClure et al., 1989).

Water activity, salt, sugar, pH, processing, packaging and atmosphere can help in reduction of the growth of pathogenic and spoilage microorganisms in RTE foods. All these together increase the hurdle rate of the product.

Currently, there is immense pressure on Food Operators to meet the FSA salt target. Hence the industry is now seeking alternates to NaCl in sea salts, KCl, $CaCl_2$ etc. But no work in locating an alternate source of salt, as an effective antimicrobial agent in terms of texture and taste, is available. The study can explore the problem, using spread matrix for example, on the consequent effect of salt reduction through replacement of the standard NaCl salt.

Challenge tests were further carried out in a general broth (MPC-broth) and with various types of salt such as NaCl, KCl and $CaCl_2$ at different levels in the liquid phase composition of the spread. It was observed that the growth of L. monocytogenes remained constant at all the salt concentrations due to the minimal nutrient present in the liquid phase. Hence as cream and buttermilk were identified as one of the high risk ingredients having a higher bacterial load it is

obvious that when they are all mixed within the product bacteria feed into them rather than the liquid phase ingredients.

One of the constraints on using various salts were with $CaCl_2$ giving a different growth curve to the others with a high initial optical density that decreased over the first 5-7 h may be as a consequence of the formation of an insoluble calcium sulphate salt. The composition of the broth contains sulphate ions that would react with the calcium chloride to produce calcium sulphate which in the course of time would settle out. After the salt settlement stage at 10 h, the growth curve was very similar to that for the other two salts hence $CaCl_2$ did not contribute any extra hurdle rate to the organism growth rate when compared with NaCl and KCl that is currently used in the industry.

As the hurdle rate for all three salts were similar, both KCl and $CaCal_2$ could be used as an alternate to NaCl to meet with the FSA reduced salt target. Hence this process could be replicated into finished products with different salt concentrations and of various types to evaluate the final salt concentration (%) and sent for further consumer testing to understand the difference in taste and texture and to repeat the challenge test in the product itself with finer droplet size rather than in the liquid phase. This can also be used to develop a growth prediction model for spread spoilage and pathogenic organisms in various salts and different product phases such as water phase or oil phase or an inert product. Only one standard spread formula (with spoilage) has been be used for this

project. The project requires further extending to other recipes such as salted, slightly salted or aerated spread produced under the SSHE.

B.cereus causes two types of food-borne illness. One type emetic disease is due to a preformed heat stable toxin whose effects are characterized by nausea, vomiting and has an incubation period of 1 to 6 hours. There is no specific limit on Bacillus cereus and other species for foods under European Community (EC) legislation. But EC legislation does require that foodstuffs should not contain microorganisms or their toxins in quantities that present an unacceptable risk for human health. Both the UK Health Protection Agency (HPA) and the Food Safety Authority Ireland (FSAI) have published guidelines on acceptable levels of microorganisms in various ready to eat foods. These require acceptable level of Bacillus cereus and other pathogenic Bacillus species in these products at $\leq 10^4$ cfu/g.

Bacillus cereus has been recognized as an agent of food poisoning since 1955. There are only a few outbreaks a year reported by CDC. Between 1972 and 1986, 52 outbreaks of food-borne diseases associated with B. cereus were reported to the CDC (two in 2003), but this is thought to represent only 2% of the total cases that have occurred during these periods. It is not a reportable disease and usually goes undiagnosed. The presence of large numbers of B. cereus (greater than 10^6 organisms/g) in a food is indicative of active growth and proliferation of the organism and is consistent with a potential hazard to health (PHE 2009).

Pasteurization of raw milk is effective in eliminating all microorganisms but the thermoduric microorganisms of the genera Microbacterium, Micrococccus, Streptococcus, Lactobacillus, Bacillus, Clostridium, the coryneforms, and occasionally some Gram-negative rods (Jay, 1996) can survive the heat treatment process. Pasteurization cannot guarantee the absence of microorganisms, when they are present in large numbers in raw milk or due to post-pasteurization contamination (Salmeron et al., 2002). The number of spores present seems to be seasonally dependent with the highest contamination occurring during the grazing period (Meer et al., 1991, Te Giffel et al., 1995). Most of the thermoduric bacteria produce extracellular proteolytic and lipolytic enzymes that are secreted into the milk are not inactivated by pasteurizing at 72°C for 25s (Griffiths et al., 1981). The residual activities of these enzymes can reduce the organoleptic quality and shelf life of processed milk products (Fairbairn et al., 1986).

This research did not look into further details of the root cause of Bacillus spp contamination in the process. Samples from the milk farms requires to be analysed and followed through the entire milk processing chain in order to find out the exact impact on the milk storage, delivery and treatment processes. A detailed microbiology challenge test approach will help to understand the effect of the presence of Bacillus spp in the shelf life of butter, spreads and milk in terms of product quality and growth potential as some psychotrophic strains are known to grow. The minimum temperature for Bacillus growth is usually 10ºC (Jay, 1996). The effect of milk collection every 2 days should also be looked into as part of the project as ingredient age played a vital role in the contaminants' level. Much

research is currently available that indicates that this affects the level of bacterial spores/toxins in fresh milk along with plant cross-contamination (Te Giffel, 1995; Salmeron et al., 2002; Ranieri et al., 2009). Different aspects to prevent Bacillus spp contamination include the farm hygiene, milk collection, storage time and temperature, CIP plant cleaning and hygiene etc all requires to be sampled and analysed along with a laboratory trial by producing emulsion with spiked Bacillus spp or Bacillus cereus in milk or cream. These completed phases will establish a control procedure on adverse effect of Bacillus spp at different seasons i.e., affect of harvesting season to the product quality, safety and spoilage.

CHAPTER 10 APPENDIX

10.1 LIST OF ACRONYMS

spp.	Species
Y&M	Yeast and Mould
FFA	Free Fatty Acid
MK	Methyl ketone
LAB	Lactic acid bacteria
V3	Variable V3 R region of rDNA
PCR	Polymerase chain reaction
API	Analytical profile index
°C	Degree Celsius
MRD	Maximum recovery diluent
l	litre
ml	mili-litre
µ	micron
µl	micro-litre
µM	micro-molar
g	grams
ng	nano-gram
mm	mili-metre
mM	mili-molar
bp	base-pair
MPCA	Milk plate count agar

TE	Tris EDTA
OGYEA	Oxy-tetracycline glucose yeast extract agar
VRBGA	Violet Red Bile Glucose Agar
TBX	Tryptone Bile X-glucuronide Medium
MKTTn	Muller-Kauffmann tetrathionate-novobiocin broth
XLD	Xylose lysine deoxycholate
PEMBA	Polymyxine-Egg yolk-Mannitol-Bacillus Agar
BPA	Baird parker agar
BHI	Brain Heart Infusion
TBA	Tributyrin agar
BPW	Buffered peptone water
MRSA	de Man Rogosa Sharpe Agar
NaOH	Sodium Hydroxide
%	‘per-cent’
v/v	volume by volume
w/v	weight by volume
cfu	Colony forming unit
UHT	Ultra high treatment
GC-MS	Gas chromatography-mass spectrometry
C8, C10, C12	Different carbon chain
FSA	Food Standard Agency
ISO	International Organisation of Standardisation
BS	British Standards
RMS	Raw Milk Silo

RCT	Reclaim Milk Tank
PA1	Pasteuriser Room
FMT	Finished Milk Tank
RTE	Ready To Eat
FBO	Food Business Operator
GMP	Good Manufacturing Process
GHP	Good Hygiene Practice

Date	Product type	Notification	Notified by	Origin	Issue	Distribution	Action	Distribution
milk and milk products								
22/01/2010	food	food poisoning	Austria	from Austria	Listeria monocytogenes (< 10 CFU/g) in syrečky cheese (Quargel Käse) from Austria	Austria, Slovakia, Poland, Germany, Czech Republic	official detention	distribution on the market (possible)
27/05/2010	food	food poisoning	Romania	from Bulgaria	Escherichia coli (between 95 and 1400 CFU/g) in cheese from Bulgaria	Romania	destruction	distribution on the market (possible)
21/04/2011	food	food poisoning	Germany	from Italy	Listeria monocytogenes (9900 CFU/g) in gorgonzola cheese from Italy	Germany	recall from consumers	distribution restricted to notifying country
17/01/2012	food	food poisoning	Belgium	from Belgium	foodborne outbreak (Salmonella Oranienburg) caused by dried milk formula from Belgium	Mozambique, Russia, Burundi, Democratic Republic of the Congo,		information on distribution not (yet) available
06/04/2012	food	food poisoning	Italy	from Germany	food poisoning suspected to be caused by semi-skimmed milk from Germany	Italy	withdrawal from the market	distribution restricted to notifying country
11/09/2012	food	food poisoning	France	from France	foodborne outbreak suspected (Salmonella Dublin) to be caused by raw milk cheese from France	Norway, Sweden, Belgium, Germany, Denmark, Nigeria, France, Spain	recall from consumers	distribution restricted to notifying country
05/09/2013	food	food poisoning	France	from France	Listeria monocytogenes (<10 CFU/g) in raw sheep's milk cheese and pasteurised milk yoghurt from France	France	recall from consumers	distribution restricted to notifying country
27/06/2014	food	food poisoning	France	from France	Salmonella kedougou in raw milk cheese Reblochon from France	France, United Kingdom, Hong Kong, Jordan, Japan, Luxembourg, New Caledonia, Nigeria, Netherlands, Philippines, Portugal, Romania, Sweden, Singapore,	withdrawal from the market	distribution to other member countries

Figure 10.1 Outbreak of pathogenic microorganisms in UK and Europe in milk and milk products since 2010 (RASFF alert)

10.3 MATERIALS AND METHODS

10.3.1 Media composition

10.3.1.1Maximum Recovery Diluent (MRD)

Maximum Recovery Diluent (MRD) supplied by Oxoid (Cat No CM 733) contains Peptone 1.0 g / l, Sodium Chloride 8.5 g /l with a final pH of 7.0 ± 0.2 at 25° C) were used throughout the study for dilution and enrichment unless stated otherwise. The diluent was made by dissolving 9.5 g of MRD in 1 litre of distilled water and was dispensed in either 9 ml or 90 ml as required into tubes and 250 ml Duran bottles and sterilised by autoclaving at 121° C for 15 minutes. When cooled it was stored in the dark in a refrigerator at 2 - 6° C or in a cupboard for up to one month. Before use it was removed from the refrigerator and allowed to come to room temperature or pre-heated to 45 ± 1° C.

10.3.1.2 Milk Plate Count Agar (MPCA)

Milk Plate Count Agar (MPCA) (CM0681 Oxoid) consists of Tryptone 5 g / l, Yeast Extract 2.5 g / l, Glucose 1 g / l, Antibiotic free skim milk 1 g / l, Agar 10 g / l at pH 6.9 ± 0.1. the agar after being inoculated was incubated at 30º C for 72 ± 4 hours (BS 4833:003). The agar was used for general microbiology count as Total Viable Count (TVC) using either spread or pour plate method.

10.3.1.3 Violet Red Bile Glucose (VRBG) Agar

Violet Red Bile Glucose (VRBG) Agar contains Yeast extract 3.0 g / l, Peptone 7.0 g / l, Sodium chloride 5.0 g / l, Bile Salts No.3 1.5 g / l, Glucose 10.0 g / l, Neutral red 0.03 g / l, Crystal violet 0.002 g / l, agar 12.0 g / l at a pH of 7.4 ± 0.2 (Oxoid CM0485). VRBGA is a selective agar to identify and isolate Enterobateriaceae family. The plates were incubated at 24 ± 2 hours at 37 ± 1°C and the purple colonies were counted.

10.3.1.4 Tryptone Bile X-glucuronide Medium (TBX)

Tryptone Bile X-glucuronide Medium (TBX) consists of Tryptone 20.0 g / l, Lactose 5.0 g / l, Bile salts No. 3 1.5 g / l, Di-potassium phosphate 4.0 g / l, Mono-potassium phosphate 1.5 g / l, Sodium chloride 5.0 g / l, 4-methylumbelliferyl-ß-D-glucuronide (MUG 0.05 g / l at pH 6.9 ± 0.2 (Oxoid CM 0979). It is a is a selective, chromogenic medium for detecting E. coli, containing the chromogen 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide, which is targeted by the enzyme glucuronidase. The plates were incubated at 37 ± 1° C for four hours and then transferred for 18 - 24 hours incubation at the selective temperature of 44 ± 0.5° C.

10.3.1.5 Buffered peptone water (BPW)

Buffered peptone water ((BPW) contains sodium chloride 5.0 g / l, disodium phosphate 3.5 g / l, monopotassium phosphate 1.5 g and final pH of 7.0.

10.3.1.6 Muller-Kauffmann tetrathionate-novobiocin broth MKTTn

Muller-Kauffmann tetrathionate-novobiocin broth MKTTn consists of Meat extract 4.3 g / l, enzymatic digest of casein 8.6 g / l, sodium chloride 2.6 g / l, calcium carbonate 38.7 g / l, sodium thiosulphate (anhydrous 30.5 g / l, Ox bile 4.78 g / l, brilliant green 0.0096 g / l at pH 8.0 ± 0.2 (Oxoid CM1048) and incubated for 24 ± 3 hours at 37 ± 1°C.

10.3.1.7 Xylose Lysine Deoxycholate Agar (XLD)

XLD medium, xylose lysine deoxycholate agar (XLD) contains yeast extract 3.0 g / l, L-Lysine HCl 5.0 g / l, xylose 3.75 g / l, lactose 7.5 g / l, sucrose 7.5 g / l, sodium desoxycholate 1.0 g / l, sodium chloride 5.0 g / l, sodium thiosulphate 6.8 g / l, ferric ammonium citrate 0.8 g / l, phenol red 0.08 g / l, agar 12.5 g / l at pH 7.4 ± 0.2 (Oxoid CM0469) and incubated at 37 $\pm$ l°C for 24 $\pm$ 3 hours.

10.3.1.8 Polymyxine Egg Yolk Mannitol Bacillus Agar (PEMBA)

Polymyxine Egg Yolk Mannitol Bacillus Agar (Oxoid CM0617) is of pH 7.2 ± 0.2 containing peptone 1.0 g / l, mannitol 10.0 g / l, sodium chloride 2.0 g / l, magnesium sulphate 0.1 g / l, disodium hydrogen phosphate 2.5 g / l, bromothymol blue 0.12 g / l, sodium pyruvate 10.0 g / l and agar 15.0 g / l and incubated at 30 ± 1°C for 72 $\pm$ 4 hours.

10.3.1.9 Mannitol Salt Agar (MSA)

Mannitol Salt Agar (MSA) contains lab-lemco' powder 1.0 g / l, peptone 10.0 g / l, mannitol 10.0 g / l, sodium chloride 75.0 g / l, phenol red 0.025 g / l, agar 15 g / l at pH 7.5 ± 0.2) and then incubated for 36 hours at 35°C.

10.3.1.10 Baird Parker agar (BPA)

Baird Parker agar (BPA) (Oxoid CM 0275), the base medium contains pancreatic digest of casein 10.0 g / l, beef extract 5.0 g / l, yeast extract 1.0 g / l, glycine 12.0 g / l, sodium pyruvate 10.0 g / l, lithium chloride 5.0 g / l and agar 20.0 g / l. Egg yolk emulsion containing potassium tellurite consists of 30% egg yolk suspension with 0.15% potassium tellurite that was added after base medium sterilization step and incubated at 37°C ± 1°C for 24 ± 2 hours.

10.3.1.11 Listeria enrichment broth

Listeria enrichment broth contains tryptone soya broth 30.0 g / l, yeast extract 6.0 g / l, potassium di-hydrogen orthophosphate 1.35 g / l, disodium hydrogen orthophosphate 9.60 g / l at pH 7.3 ± 0.2 (Oxoid CM 0897) and enriched for two days at 30 ± 1° C.

10.3.1.12 Listeria selective agar Oxford

Listeria selective agar Oxford contains Columbia Blood Agar base 39.0 g / l, aesculin 1.0 g / l, ferric ammonium citrate 0.5 g / l, lithium chloride 15.0 g / l at

pH 7.0 ± 0.2 (CM 0856) where Listeria selective supplement was added (one vial per 500 ml medium) (cycloheximide 200 mg, colistin sulphate 10.0 mg, acriflavine 2.5 mg, cefotetan 1.0 mg, fosfomycin 5.0 mg) (SR 0140).

10.3.1.13 C-F-C Pseudomonas agar

C-F-C Pseudomonas agar contains Gelatin peptone 16.0 g / l, Casein hydrolysate 10.0 g / l, Potassium sulphate 10.0 g / l, Magnesium chloride 1.4 g / l and agar 11.0 g / l with pH 7.1 ± 0.2 (CM 0559). The C-F-C agar supplement contains Cetrimide 5.0 mg, Fucidin 5.0 mg, Cephalosporin 25.0 mg added one vial per 500ml of agar (SR 0103). The plates were incubated at 25 ± 1°C for 48 $\pm$ 4 hours.

10.3.1.14 Caseinate Medium

The Caseinate Medium contains Casein enzymic hydrolysate 5.0 g / l, Yeast extract 2.5 g / l, Dextrose 1.0 g / l, Sodium Caseinate 10.0 g / l, Trisodium citrate 4.410 g / l and Calcium chloride 2.220 g / l and 15 g / l Agar (Oxoid, CM1029).

10.3.1.15 Tributyrin Agar (TBA)

Tributyrin Agar (TBA) contains Peptic digest of animal tissue 5.0 g / l, Yeast extract 3.0 g / l, Agar 15.0 g / l and a final pH of 7.5±0.2 (Oxoid, CM0075B) incubated at 30 ± 1°C for 72 $\pm$ 4 hours.

10.3.1.16 MRSA (de Man, Rogosa, Sharpe Agar)

MRSA (de Man, Rogosa, Sharpe Agar) contains peptone 10.0 g / l, `lab-lemco' powder 8.0 g / l, yeast extract 4.0 g / l, glucose 20.0 g / l, sorbitan mono-oleate 1 ml / l, dipotassium hydrogen phosphate 2.0 g / l, sodium acetate $3H_2O$ 5.0 g / l, triammonium citrate 2.0 g / l, magnesium sulphate $7H_2O$ 0.2 g / l, manganese sulphate $4H_2O$ 0.05 g / l, agar 10.0 g / l with a pH 6.2 ± 0.2 (CM0361) is a non-selective medium designed for the enumeration of lactic acid bacteria.

10.3.1.17 Czapek Dox Agar

Czapek Dox Agar (modified) (Oxoid CM0097) contains sodium nitrate 2.0 g / l, potassium chloride 0.5 g / l, magnesium glycerophosphate 0.5 g / l, ferrous sulphate 0.01 g / l, potassium sulphate 0.35 g / l, sucrose 30.0 g / l, agar 12.0 g / l and pH pH 6.8 ± 0.2 is a solid defined medium for the cultivation of fungi that are able to utilise sodium nitrate as the sole source of nitrogen. For the broth the same formulation was used without the agar.

10.3.1.18 Oxytetracyline Glucose Yeast Extract Agar (OGYEA)

Oxytetracyline Glucose Yeast Extract Agar (OGYEA) (Oxoid CM0545) containing yeast extract 5.0 g / l, glucose 20.0 g / l, agar 12.0 g / l and pH 7.6 ± 0.2) (one vial of oxytetracyline selective supplement Oxoid SR0073 was added per 500 ml of medium) (selective agar was used as it inhibits the bacterial growth.

10.4. RESULTS

Table 10.1 Summary of the total number of microorganisms enumerated from 100 products (Complaint samples 1-85, Fresh product 86-100)

ID	Counts (cfu / g)									
	Total Count	**Enterobacteriaceae**	**Pseudomonas spp**	**Proteolytic Bacteria**	**Lactic Acid Bacteria**	**Lipolytic Bacteria**	**Bacillus spp**	**Staphylococcus spp**	**Yeast**	**Mould**
1	450	<1	<1	<1	<1	64	100	150	10	73
2	402	<1	<1	<1	<1	42	300		50	10
3	296	<1	<1	<1	<1	65	86	120	13	12
4	820	25	<1	<1	<1	234	150	300	23	32
5	128	<1	<1	<1	<1	54	<1	<1	21	53
6	1550	63	<1	<1	<1	523	300	250	13	67
7	450	<1	<1	<1	<1	69	88	40	244	14
8	283	<1	<1	<1	<1	42	120	55	43	23
9	620	<1	<1	<1	<1	68	220	150	24	77
10	64	<1	<1	<1	<1	37	<1	<1	23	4
11	580	<1	<1	<1	<1	89	20	52	234	22
12	270	<1	<1	<1	<1	89	84	180	23	100
13	190	<1	<1	<1	<1	48	32	20	45	45
14	261	<1	<1	<1	<1	47	110	56	43	5
15	35000	2500	120	1020	248	1500	20000	950	46	6
16	309	<1	<1	<1	<1	28	67	124	56	34
17	550	<1	<1	<1	<1	70	<1	<1	345	34
18	251	<1	<1	<1	<1	67	30	55	65	34
19	61	<1	<1	<1	<1	20	<1	<1	34	7
20	1850	<1	<1	<1	<1	520	300	550	45	120
21	2500	178	50	<1	<1	980	300	300	54	69
22	114	<1	<1	<1	<1	40	<1	<1	29	45
23	920	<1	<1	<1	<1	29	300	300	32	69
24	332	<1	<1	<1	<1	100	80	60	47	45
25	760	<1	<1	<1	<1	78	250	120	129	200
26	3520	190	46	<1	<1	690	1200	250	23	156
27	120	<1	<1	<1	<1	35	<1	<1	65	20
28	680	<1	<1	<1	<1	184	254	58	23	43
29	1800	255	39	<1	<1	550	350	270	56	43
30	32	<1	<1	<1	<1	32	<1	<1	<1	<1
31	570	<1	<1	<1	<1	286	80	60	74	70
32	7100	1260	450	380	255	1894	2500	280	38	49
33	220	<1	<1	<1	<1	<1	<1	<1	34	120
34	45000	10400	1200	1020	32	25000	350000	300	23	56
35	4200	<1	<1	<1	<1	1560	1800	620	34	89
36	2020	<1	<1	<1	<1	830	670	350	84	93
37	820	<1	<1	<1	<1	340	248	120	37	20
38	234	<1	<1	<1	<1	234	<1	<1	<1	<1
39	500	<1	<1	<1	<1	264	110	80	23	24
40	80	<1	<1	<1	<1	<1	<1	<1	76	4

ID	Counts (cfu / g)									
	Total Count	**Enterobacteriaceae**	**Pseudomonas spp**	**Proteolytic Bacteria**	**Lactic Acid Bacteria**	**Lipolytic Bacteria**	**Bacillus spp**	**Staphylococcus spp**	**Yeast**	**Mould**
41	3200	560	220	<1	<1	920	850	90	37	20
42	430	<1	<1	<1	<1	435	<1	<1	<1	<1
43	400	<1	<1	<1	<1	186	88	25	46	47
44	52	<1	<1	<1	<1	52	<1	<1	<1	<1
45	266	<1	<1	<1	<1	125	50	30	39	22
46	40500	11100	950	1000	245	1850	22000	860	20	23
47	4000	980	243	<1	<1	942	1500	260	27	43
48	237	<1	<1	<1	<1	237	<1	<1	<1	<1
49	3200	256	21	<1	<1	1150	1020	300	23	32
50	47	<1	<1	<1	<1	47	<1	<1	<1	<1
51	306	<1	<1	<1	<1	120	80	46	40	20
52	0	<1	<1	<1	<1	<1	<1	<1	<1	<1
53	2200	356	78	<1	<1	620	880	220	24	24
54	86	<1	<1	<1	<1	<1	<1	<1	23	63
55	1080	<1	<1	<1	<1	530	300	150	47	57
56	1020	<1	<1	<1	<1	300	250	320	37	76
57	1030	<1	<1	<1	<1	320	180	450	26	39
58	400	<1	<1	<1	<1	100	100	120	49	37
59	1050	<1	<1	<1	<1	380	300	280	58	30
60	780	<1	<1	<1	<1	138	150	330	48	30
61	1550	<1	<1	<1	<1	193	300	560	245	85
62	5500	1200	2140	150	13	169	200	1000	34	84
63	1020	<1	<1	<1	<1	233	250	300	47	63
64	880	<1	<1	<1	<1	125	150	280	37	83
65	660	<1	<1	<1	<1	132	200	140	25	47
66	650	<1	<1	<1	<1	164	80	300	37	53
67	1000	<1	<1	<1	<1	257	300	360	39	36
68	760	<1	<1	<1	<1	274	300	80	28	83
69	850	<1	<1	<1	<1	790	<1	<1	23	39
70	3580	780	289	<1	<1	900	1250	300	18	36
71	300	<1	<1	<1	<1	88	60	38	28	63
72	62	<1	<1	<1	<1	<1	<1	<1	35	27
73	2800	267	21	<1	<1	720	1420	250	28	64
74	338	<1	<1	<1	<1	59	84	10	129	56
75	223	<1	<1	<1	<1	29	78	44	38	34
76	216	173	<1	<1	<1	43	<1	<1	<1	<1
77	2550	<1	<1	24	23	860	1100	88	120	34
78	208	<1	<1	<1	<1	21	100	24	19	44
79	329	<1	<1	<1	<1	23	118	90	10	88
80	261	<1	<1	<1	<1	57	50	40	16	98
81	261	<1	<1	<1	<1	73	56	38	18	76
82	454	<1	<1	22	3	422	<1	<1	<1	<1
83	780	213	21	<1	<1	31	236	78	45	48
84	820	45	19	<1	<1	57	576	10	24	42

ID	Counts (cfu / g)									
	Total Count	**Enterobacteriaceae**	**Pseudomonas spp**	**Proteolytic Bacteria**	**Lactic Acid Bacteria**	**Lipolytic Bacteria**	**Bacillus spp**	**Staphylococcus spp**	**Yeast**	**Mould**
85	0	<1	<1	<1	<1	<1	<1	<1	<1	<1
86	<10	<1		<1	<1	<1	<1	<1	<1	<1
87	20	<1	10	<1	<1	<1	<1	<1	<1	<1
88	50	20		<1	<1	<1	<1	<1	<1	<1
89	100	<1	50	<1	100	<1	100	110	<1	<1
90	<10	<1		<1	<1	<1	<1	<1	<1	<1
91	15	<1	12	<1	<1	<1	<1	<1	<1	<1
92	<10	<1	<1	<1	<1	<1	<1	<1	<1	<1
93	52	<1	<1	<1	25	<1	10	<1	<1	<1
94	<10	<1	<1	<1	<1	<1	<1	<1	<1	<1
95	<10	<1	2	<1	<1	<1	<1	<1	<1	<1
96	<10	<1	<1	<1	<1	<1	<1	<1	<1	<1
97	<10	<1	<1	<1	<1	<1	<1	<1	<1	<1
98	<10	<1	10	<1	<1	<1	<1	<1	<1	<1
99	<10	<1	<1	<1	<1	<1	<1	<1	<1	<1
100	<10	<1	<1	<1	<1	<1	<1	<1	<1	<1

10.5 CHEMICAL ANALYSIS

Table 10.2 Summary of sensory testing in 'cheese flavour', standard and market spreads at 5ºC and 20ºC.

Sample Number	Standard Samples						Cheese Flavour Samples (End of life)		Market Samples					
	Start of Life (Day 1)		Middle of Life (Day 50)		End of Life (Day 100)				Start of Life (Day 1)		Middle of Life (Day 50)		End of Life (Day 100)	
	5ºC	20ºC	5ºC	20ºC	5ºC	20ºC	5ºC	20ºC	5ºC	20ºC	5ºC	20ºC	5ºC	20ºC
1	ND	ND	ND	ND	ND	ND	ND	B3	ND	ND	ND	ND	ND	ND
2	ND	B1	ND	B2	ND	B2	B1	B2	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND	ND	B3	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND	ND	B2	B1	ND	ND	ND	ND	ND	ND
5	ND	ND	ND	ND	ND	ND	ND	B1	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	B2	ND	B2	B2	B3	ND	ND	ND	ND	ND	ND
7	ND	ND	ND	ND	ND	ND	B2	B1	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	ND	B2	ND	B2	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND	ND	B1	B1	ND	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND	ND	B1	B1	ND	ND	ND	ND	ND	ND
11	ND	ND	ND	ND	ND	ND	ND	B3						
12	ND	B1	ND	B2	ND	B2	B1	B1						
13	ND	ND	ND	ND	ND	ND	ND	B3						
14	ND	ND	ND	ND	ND	ND	ND	B1						
15	ND	ND	ND	ND	ND	ND	B1	B3						
16	ND	ND	ND	ND	ND	B2	ND	B3						
17	ND	ND	ND	ND	ND	ND	B1	B2						
18	ND	ND	ND	B2	ND	ND	B3	B3						
19	ND	B1	ND	B2	ND	B2	ND	B3						
20	ND	ND	ND	ND	ND	ND	B2	B1						
21	ND	ND	ND	ND	ND	ND	ND	B3						
22	ND	ND	ND	ND	ND	ND	B3	ND						
23	ND	ND	ND	ND	ND	ND	B1	B2						
24	ND	ND	ND	ND	ND	ND	B2	B1						
25	ND	B1	ND	B2	ND	B2	ND	B3						
26	ND	ND	ND	ND	ND	ND	ND	B3						
27	ND	ND	ND	ND	ND	ND	B3	B2						
28	ND	ND	ND	ND	ND	ND	B1	B1						
29	ND	B1	ND	B2	ND	B2	B3	B3						
30	ND	ND	ND	ND	ND	ND	B2	B3						
31	ND	ND	ND	ND	ND	ND	B3	B2						
32	ND	ND	ND	ND	ND	ND	B1	B3						
33	ND	B1	ND	B2	ND	B2	B2	B2						
34	ND	ND	ND	ND	ND	ND	B3	B3						
35	ND	ND	ND	ND	ND	ND	B1	B3						
36	ND	ND	ND	ND	ND	ND	B3	B2						
37	ND	B1	ND	B2	ND	B2	B2	B3						
38	ND	ND	ND	ND	ND	ND	B1	ND						
39	ND	ND	ND	ND	ND	ND	B2	B2						
40	ND	ND	ND	ND	ND	ND	B3	B1						
41	ND	ND	ND	ND	ND	B2	B3	B3						
42	ND	ND	ND	ND	ND	ND	B1	ND						
43	ND	B1	ND	B2	ND	B2	ND	B2						
44	ND	ND	ND	ND	ND	ND	B2	B3						
45	ND	B1	ND	B2	ND	B2	ND	B3						
46	ND	ND	ND	ND	ND	ND	B1	ND						
47	ND	B1	ND	B1	ND	B2	B2	B2						
48	ND	B1	ND	B2	ND	B2	B2	B1						
49	ND	B1	ND	B2	ND	B2	B1	B3						
50	ND	B1	ND	B2	ND	B2	B3	B1						

10.6 CHEMICAL CHALLNEGE TEST

Table 10.3 Challenge test of spread ingredients with the causative organisms (Bacillus spp, Staphylococcus spp, Y&M)

DETAILS		LEVEL OF METHYL ETHYL KETONE DETECTION (mg/kg)				
		TOTAL	2-PENTANONE (C6)	2-HEPTANONE (C8)	2-NONANONE (C10)	2-UNDECANONE (C12)
Skimmed Milk	Control	0.02	<0.01	0.02	<0.02	0.05
Skimmed Milk	*Bacillus* spp	<0.01	<0.01	<0.02	0.02	<0.01
Skimmed Milk	*Bacillus* spp	0.02	<0.01	<0.02	0.02	<0.01
Skimmed Milk	*Bacillus* spp	0.01	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	*Bacillus* spp	0.02	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	*Bacillus* spp	0.02	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	*Staphylococcus* spp	<0.01	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	*Staphylococcus* spp	0.02	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	*Staphylococcus* spp	<0.01	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	*Staphylococcus* spp	0.01	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	*Staphylococcus* spp	0.01	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	Yeast	0.01	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	Yeast	0.01	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	Yeast	0.01	<0.01	0.05	0.06	<0.01
Skimmed Milk	Yeast	<0.01	<0.01	<0.02	0.03	<0.01
Skimmed Milk	Yeast	<0.01	<0.01	<0.02	0.03	<0.01
Skimmed Milk	Yeast	0.02	<0.01	<0.02	0.03	<0.01
Skimmed Milk	Yeast	0.02	<0.01	<0.02	0.03	<0.01
Skimmed Milk	Mould	0.01	0.01	0.14	0.14	<0.01
Skimmed Milk	Mould	0.02	<0.01	<0.02	<0.02	<0.01
Skimmed Milk	Mould	0.01	<0.01	0.12	0.12	<0.01
Cream	Control	0.02	<0.01	0.03	0.26	0.26
Cream	*Bacillus* spp	0.01	0.01	<0.02	0.02	0.02
Cream	*Bacillus* spp	0.01	0.03	0.07	<0.01	<0.01
Cream	*Bacillus* spp	0.02	0.02	0.11	<0.01	<0.01
Cream	*Bacillus* spp	0.01	0.04	0.12	<0.01	<0.01
Cream	*Bacillus* spp	0.01	0.01	0.05	<0.01	<0.01
Cream	*Staphylococcus* spp	0.02	<0.01	0.08	<0.01	<0.01
Cream	*Staphylococcus* spp	0.02	0.01	0.09	<0.01	<0.01
Cream	*Staphylococcus* spp	0.01	0.03	<0.02	0.04	0.04
Cream	*Staphylococcus* spp	0.01	0.02	0.06	<0.01	<0.01
Cream	*Staphylococcus* spp	0.01	<0.01	<0.02	<0.01	<0.01
Cream	Yeast	0.01	0.02	0.07	<0.01	<0.01
Cream	Yeast	0.02	0.01	0.03	<0.01	<0.01
Cream	Yeast	<0.01	0.01	0.04	<0.01	<0.01
Cream	Yeast	0.02	0.03	0.14	0.01	0.01
Cream	Yeast	0.01	0.04	0.08	<0.01	<0.01
Cream	Mould	0.01	<0.01	<0.02	<0.01	<0.01
Cream	Mould	0.01	0.02	0.08	<0.01	<0.01
Cream	Mould	0.01	0.31	1.08	0.02	0.02
Cream	Mould	0.01	<0.01	0.11	0.02	0.02
Cream	Mould	<0.01	0.01	0.08	<0.01	<0.01
Buttermilk	Control	0.01	<0.01	0.03	0.04	0.08
Buttermilk	*Bacillus* spp	0.01	0.04	0.12	<0.01	<0.01
Buttermilk	*Bacillus* spp	0.01	0.01	0.05	<0.01	<0.01
Buttermilk	*Bacillus* spp	0.01	<0.01	0.07	0.02	0.02
Buttermilk	*Bacillus* spp	0.01	0.01	0.07	<0.01	<0.01
Buttermilk	*Bacillus* spp	0.01	0.04	<0.02	0.02	0.02
Buttermilk	*Staphylococcus* spp	0.01	0.02	0.05	<0.01	<0.01
Buttermilk	*Staphylococcus* spp	0.02	<0.01	0.08	<0.01	<0.01
Buttermilk	*Staphylococcus* spp	0.02	0.01	0.09	<0.01	<0.01
Buttermilk	*Staphylococcus* spp	0.01	0.03	<0.02	0.04	0.04
Buttermilk	*Staphylococcus* spp	0.01	0.02	0.06	<0.01	<0.01
Buttermilk	Yeast	0.02	<0.01	<0.02	0.03	<0.01
Buttermilk	Yeast	<0.01	<0.01	<0.02	<0.02	0.01
Buttermilk	Yeast	<0.01	0.01	<0.02	0.04	<0.01
Buttermilk	Yeast	0.01	0.02	0.07	<0.01	<0.01
Buttermilk	Yeast	0.02	0.01	0.03	<0.01	<0.01
Buttermilk	Mould	0.01	<0.01	<0.02	0.02	0.54
Buttermilk	Mould	<0.01	0.01	<0.02	<0.02	0.01
Buttermilk	Mould	<0.01	<0.01	<0.02	0.02	1.54
Buttermilk	Mould	0.01	<0.01	<0.02	0.02	0.54
Buttermilk	Mould	<0.01	0.01	<0.02	<0.02	0.01
Oils 1	*Bacillus* spp	<0.01	<0.01	<0.02	<0.02	<0.01
Oils 1	*Bacillus* spp	0.02	0.03	0.03	<0.02	<0.01
Oils 1	*Bacillus* spp	0.01	<0.01	<0.02	<0.02	<0.01
Oils 1	*Staphylococcus* spp	<0.01	<0.01	<0.02	<0.02	<0.01
Oils 1	*Staphylococcus* spp	0.02	<0.01	<0.02	<0.02	<0.01
Oils 1	*Staphylococcus* spp	0.01	<0.01	<0.02	<0.02	<0.01
Oils 1	Yeast	<0.01	0.01	<0.02	<0.02	<0.01
Oils 1	Yeast	<0.01	<0.01	<0.02	<0.02	<0.01
Oils 1	Yeast	0.01	<0.01	<0.02	0.03	<0.01
Oils 1	Mould	<0.01	<0.01	<0.02	<0.02	<0.01
Oils 1	Mould	0.01	<0.01	<0.02	0.02	1.01
Oils 1	Mould	0.01	<0.01	<0.02	<0.02	<0.01
Oils 2	*Bacillus* spp	0.03	0.03	0.03	<0.02	<0.01
Oils 2	*Bacillus* spp	0.02	<0.01	<0.02	<0.02	<0.01
Oils 2	*Bacillus* spp	0.02	0.03	0.02	<0.02	<0.01
Oils 2	*Staphylococcus* spp	0.01	0.02	<0.02	<0.02	<0.01
Oils 2	*Staphylococcus* spp	0.02	0.04	0.04	<0.02	<0.01
Oils 2	*Staphylococcus* spp	0.01	<0.01	<0.02	0.02	<0.01
Oils 2	Yeast	0.02	0.04	0.06	0.05	<0.01
Oils 2	Yeast	<0.01	<0.01	<0.02	<0.02	0.01
Oils 2	Yeast	<0.01	0.01	<0.02	0.04	<0.01
Oils 2	Mould	<0.01	0.01	<0.02	<0.02	0.01
Oils 2	Mould	0.01	<0.01	<0.02	<0.02	1.17
Oils 2	Mould	<0.01	<0.01	<0.02	<0.02	<0.01
Oils 3	*Bacillus* spp	0.02	0.01	0.04	0.02	<0.01
Oils 3	*Staphylococcus* spp	<0.01	<0.01	<0.02	<0.02	<0.01
Oils 3	*Staphylococcus* spp	0.02	0.02	0.03	0.02	<0.01
Oils 3	*Staphylococcus* spp	<0.01	<0.01	<0.02	<0.02	<0.01
Oils 3	Yeast	0.01	0.02	0.03	<0.02	<0.01
Oils 3	Yeast	0.01	<0.01	<0.02	<0.02	<0.01
Oils 3	Yeast	<0.01	<0.01	<0.02	<0.02	<0.01
Oils 3	Mould	<0.01	<0.01	<0.02	<0.02	<0.01
Oils 3	Mould	0.01	<0.01	<0.02	<0.02	1.17
Oils 3	Mould	<0.01	<0.01	<0.02	<0.02	<0.01

10.7 MICROBIAL CHEMICAL CHALLENGE TEST

Table 10.4 Summary of isolates identified from the dairy environment and their genetic similarity

Factory	Sample No	Description	Identified Species
A	4860308	Floor	*L. monocytogenes*
	4857461	Pasteuriser	
	4860334	Conveyor	
	4839172	Floor	
	4860462	Product	
	4860408	Filler	
	4860398	Filler	
	4860404	Filler	
	4860323	Filler	
	4861531	Filler	
	4860364	Drain	
	4929174	Drain	
	4862536a	Drain	
	4862536b	Drain	
	4842733	Drain	
	4773620	Process	
	4805099	Process	
	4880909	RCT	
B	4908986	Raw Milk Tankers	*L. monocytogenes*
	4908908	PA3	
	4817604	Raw Milk Silos	
	4817601	Raw Milk Silos	
	4817605	Raw Milk Silos	
	4817603	Raw Milk Silos	
	4817602	Raw Milk Silos	
	4817645 repeat	Raw Milk Silos	
	4807767	Process	
	4809328	Floor	
	4817645	Process	
C	4800222	Floor	*L. innocua*
	4800221	Floor	
	4820583	Contractors Locker Room	
	4820582	Air Lock Room	
D	4830762	Conveyor	*L. monocytogenes*
	4829451	Conveyor	
E	4860396	Floor	*L. monocytogenes*
	4862579	Floor	
	Reference control	Laboratories reference control	
	4802031 Repeat	Floor	
F	4906474	Raw Milk Tankers	*L. monocytogenes*
	4906475	Raw Milk Tankers	*L. innocua*
	4906478	Raw Milk Tankers	*L. monocytogenes*
	4906478	Raw Milk Tankers	*L. monocytogenes*
	4847548	Finished Milk Tanks	*L. monocytogenes*
	4936221	Conveyor	*L. welshimerii*
	4871314	Path lab confirmation bench	*Lab strain*
	4807777	Floor	*L. innocua*
	4804897 Repeat	Floor	*L. innocua*

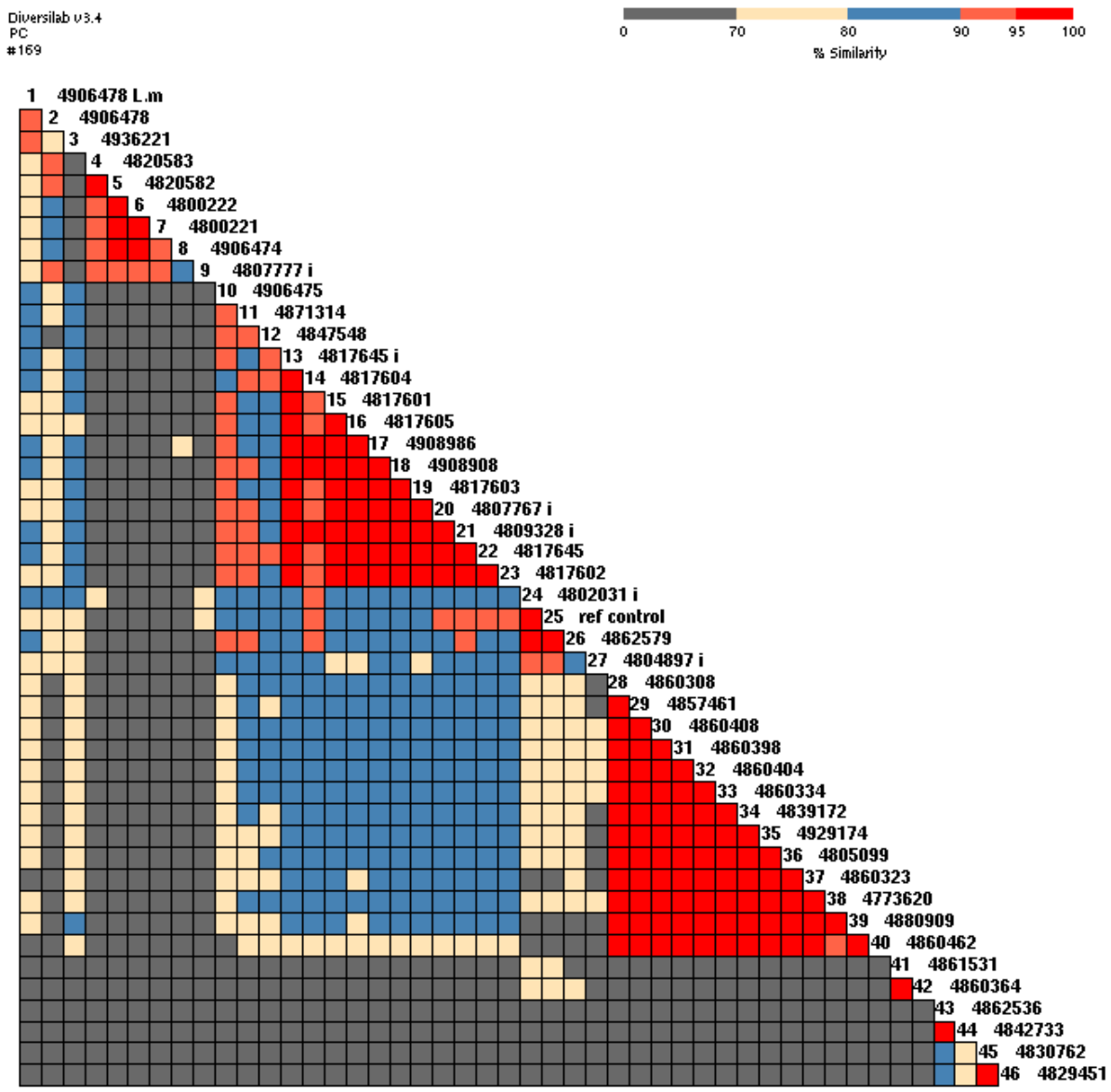


Figure 10.2 The percentage relationship between the samples in the experiment. There are seven distinctive groups , where some isolates are genetically identical and some are genetically similar.

Table 10.5 Listeria spp growth curve in various broth (MPC Broth, Unsalted liquid phase and unsalted liquid phase with potassium sorbate

Generation time (hrs)	Unsalted no K sorbate Liquid Phase		Unsalted K sorbate Liquid Phase		MPC Broth	
	G1	G2	G1	G2	G1	G2
0	0.031	0.033	0.031	0.032	0.123	0.121
1	0.031	0.033	0.031	0.033	0.127	0.126
2	0.031	0.033	0.031	0.033	0.157	0.153
3	0.034	0.037	0.030	0.032	0.222	0.206
4	0.036	0.038	0.031	0.032	0.328	0.290
5	0.037	0.039	0.031	0.033	0.457	0.473
6	0.036	0.039	0.031	0.033	0.499	0.697
7	0.041	0.044	0.031	0.033	0.510	0.700
8	0.041	0.044	0.031	0.032	0.509	0.691
9	0.052	0.055	0.031	0.032	0.500	0.689
10	0.062	0.064	0.032	0.033	0.506	0.674
11	0.065	0.067	0.031	0.033	0.506	0.664
12	0.072	0.074	0.032	0.032	0.508	0.658
13	0.073	0.076	0.032	0.032	0.504	0.652
14	0.079	0.082	0.032	0.033	0.501	0.648
15	0.096	0.099	0.033	0.033	0.502	0.645
16	0.132	0.135	0.032	0.033	0.498	0.643
17	0.156	0.160	0.033	0.033	0.498	0.638
18	0.168	0.171	0.033	0.033	0.492	0.637
19	0.178	0.182	0.033	0.033	0.492	0.633
20	0.180	0.184	0.032	0.033	0.493	0.630
21	0.185	0.189	0.032	0.033	0.489	0.628
22	0.199	0.203	0.032	0.033	0.488	0.625
23	0.234	0.238	0.032	0.033	0.483	0.622
24	0.279	0.284	0.032	0.033	0.480	0.619

Figure 10.3 G1 L. monocytogenes growth replicates at various concentrations of NaCl, KCl and $CaCl_2$

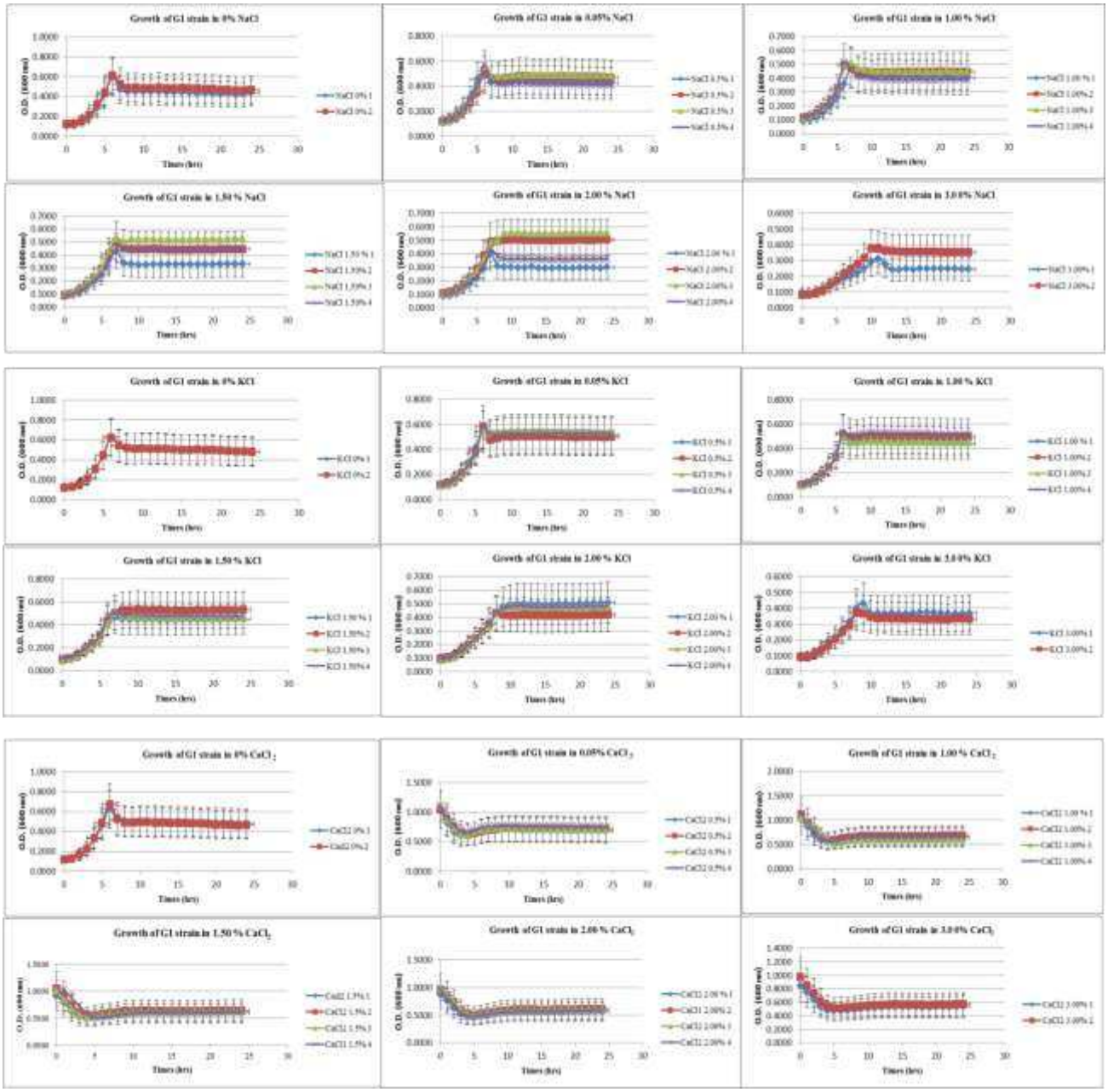


Figure 10.4 G2 L. monocytogenes growth replicates at various concentrations of NaCl, KCl and $CaCl_2$

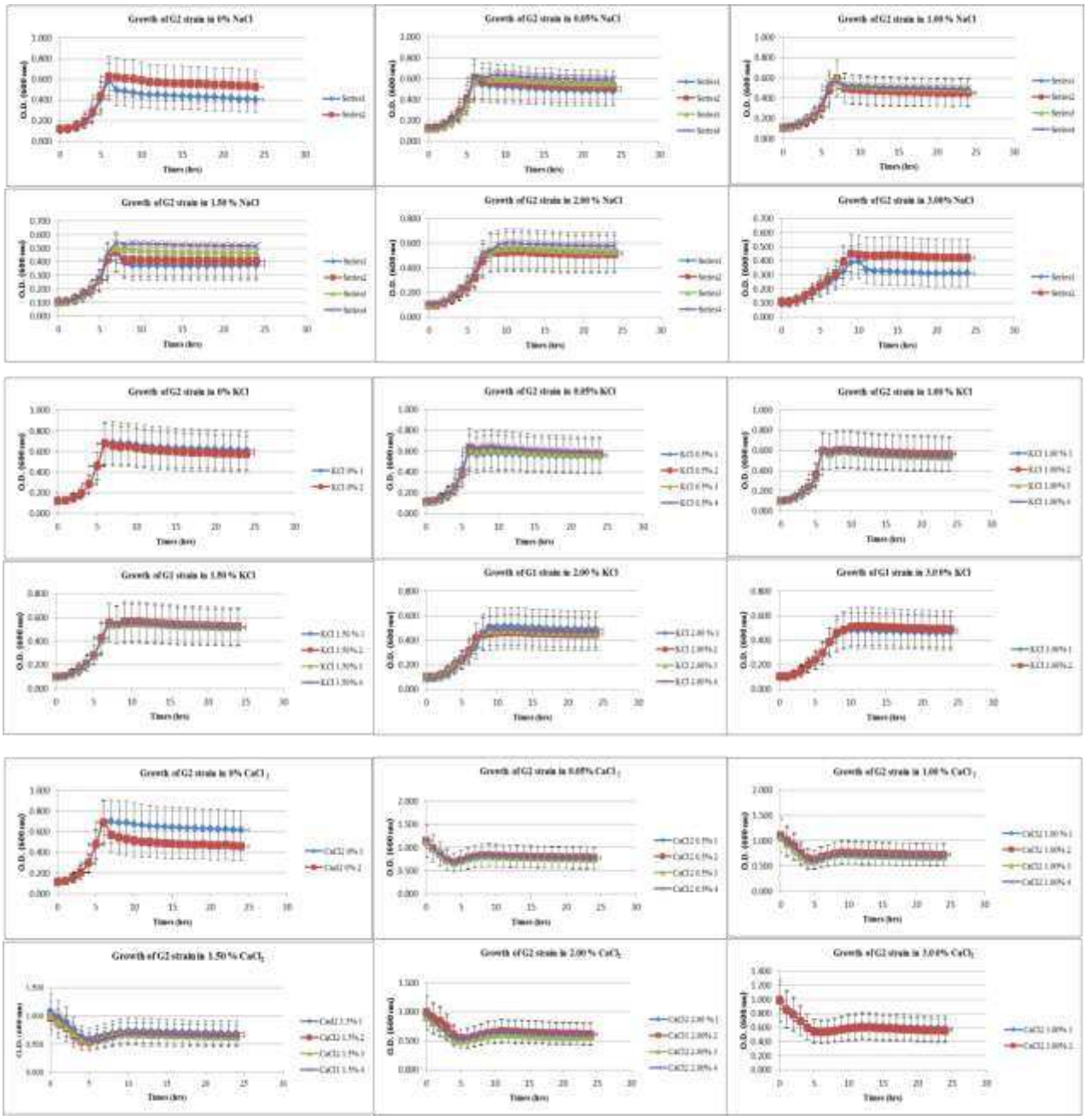


Table 10.6 G1 L. monocytogenes growth in MPC broth with various levels of NaCl

	NaCl																							
	0.00%			0.50%					1.00%					1.50%					2.00%					3.00%		
Time	B2	C2	Average	B3	C3	C4	B4	Average	B5	C5	C6	B6	Average	B7	C7	C8	B8	Average	B9	C9	C10	B10	Average	B11	C11	Average
0	0.1051	0.1258	0.115	0.1285	0.1166	0.1182	0.1178	0.120	0.0889	0.1137	0.1164	0.1138	0.108	0.1019	0.0907	0.1064	0.0952	0.099	0.0906	0.1075	0.1074	0.0976	0.101	0.1003	0.0816	0.091
1	0.1158	0.1269	0.121	0.1380	0.1269	0.1290	0.1284	0.131	0.1002	0.1227	0.1238	0.1239	0.118	0.1056	0.1021	0.1141	0.1025	0.106	0.0971	0.1127	0.1078	0.1026	0.105	0.0951	0.0849	0.090
2	0.1447	0.1609	0.153	0.1711	0.1539	0.1581	0.1563	0.160	0.1186	0.1494	0.1486	0.1509	0.142	0.1243	0.1284	0.1391	0.1239	0.129	0.1143	0.1346	0.1288	0.1219	0.125	0.1048	0.0927	0.099
3	0.1975	0.2195	0.209	0.2293	0.2076	0.2155	0.2130	0.216	0.1509	0.1927	0.1941	0.1985	0.184	0.1592	0.1777	0.1811	0.1594	0.169	0.1495	0.1752	0.1682	0.1590	0.163	0.1245	0.1102	0.117
4	0.2852	0.3151	0.300	0.3087	0.2686	0.2915	0.2951	0.291	0.1974	0.2419	0.2544	0.2534	0.237	0.1985	0.2289	0.2360	0.2076	0.218	0.1785	0.2265	0.2211	0.1946	0.205	0.1400	0.1428	0.141
5	0.4214	0.4437	0.433	0.4536	0.3562	0.3879	0.4010	0.400	0.2620	0.3257	0.3328	0.3339	0.314	0.2485	0.2969	0.3003	0.2520	0.274	0.2229	0.2885	0.2762	0.2371	0.256	0.1628	0.1753	0.169
6	0.5951	0.6129	0.604	0.4895	0.5239	0.5608	0.5732	0.537	0.3584	0.4957	0.4993	0.5130	0.467	0.3764	0.4110	0.4315	0.3353	0.389	0.2940	0.3860	0.3540	0.3071	0.335	0.1835	0.2159	0.200
7	0.4710	0.5275	0.499	0.4289	0.4609	0.4770	0.4453	0.453	0.4823	0.4718	0.5112	0.4658	0.483	0.4326	0.5038	0.5316	0.4635	0.483	0.3986	0.4860	0.4807	0.4329	0.450	0.2004	0.2451	0.223
8	0.4463	0.4893	0.468	0.4317	0.4568	0.4822	0.4141	0.446	0.4207	0.4378	0.4768	0.4096	0.436	0.3412	0.4579	0.5084	0.4402	0.437	0.3082	0.4969	0.5020	0.3814	0.422	0.2234	0.2782	0.251
9	0.4468	0.4895	0.468	0.4311	0.4671	0.4823	0.4136	0.449	0.4157	0.4474	0.4641	0.4081	0.434	0.3331	0.4484	0.5270	0.4437	0.438	0.3024	0.5029	0.5526	0.3663	0.431	0.2508	0.3179	0.284
10	0.4473	0.4812	0.464	0.4296	0.4694	0.4906	0.4213	0.453	0.4062	0.4429	0.4556	0.3945	0.425	0.3268	0.4483	0.5230	0.4449	0.436	0.3006	0.5052	0.5563	0.3603	0.431	0.2930	0.3793	0.336
11	0.4466	0.4844	0.466	0.4319	0.4805	0.4933	0.4252	0.458	0.4131	0.4424	0.4526	0.3912	0.425	0.3278	0.4463	0.5259	0.4422	0.436	0.2987	0.5074	0.5562	0.3617	0.431	0.3092	0.3740	0.342
12	0.4490	0.4903	0.470	0.4316	0.4787	0.4910	0.4224	0.456	0.4104	0.4444	0.4523	0.3887	0.424	0.3299	0.4510	0.5238	0.4432	0.437	0.2979	0.5045	0.5568	0.3636	0.431	0.2760	0.3629	0.319
13	0.4431	0.4809	0.462	0.4321	0.4763	0.4917	0.4189	0.455	0.4064	0.4407	0.4570	0.3878	0.423	0.3309	0.4472	0.5234	0.4443	0.436	0.3037	0.5011	0.5535	0.3613	0.430	0.2411	0.3585	0.300
14	0.4437	0.4836	0.464	0.4328	0.4759	0.4936	0.4149	0.454	0.4094	0.4461	0.4527	0.3888	0.424	0.3323	0.4442	0.5232	0.4429	0.436	0.2951	0.5017	0.5546	0.3616	0.428	0.2415	0.3566	0.299
15	0.4412	0.4878	0.465	0.4301	0.4730	0.4919	0.4175	0.453	0.4083	0.4447	0.4499	0.3843	0.422	0.3326	0.4443	0.5207	0.4423	0.435	0.2937	0.5011	0.5532	0.3601	0.427	0.2483	0.3570	0.303
16	0.4390	0.4813	0.460	0.4300	0.4750	0.4889	0.4174	0.453	0.4085	0.4455	0.4519	0.3899	0.424	0.3311	0.4445	0.5211	0.4410	0.434	0.2926	0.5006	0.5534	0.3565	0.426	0.2439	0.3555	0.300
17	0.4297	0.4808	0.455	0.4294	0.4776	0.4918	0.4156	0.454	0.4063	0.4444	0.4504	0.3878	0.422	0.3310	0.4442	0.5225	0.4417	0.435	0.2970	0.5031	0.5544	0.3619	0.429	0.2495	0.3556	0.303
18	0.4323	0.4775	0.455	0.4268	0.4759	0.4913	0.4141	0.452	0.4058	0.4449	0.4498	0.3863	0.422	0.3312	0.4456	0.5245	0.4407	0.436	0.2933	0.5030	0.5556	0.3566	0.427	0.2473	0.3561	0.302
19	0.4269	0.4764	0.452	0.4259	0.4730	0.4894	0.4139	0.451	0.4060	0.4445	0.4501	0.3857	0.422	0.3319	0.4464	0.5236	0.4412	0.436	0.2942	0.5037	0.5573	0.3613	0.429	0.2478	0.3557	0.302
20	0.4248	0.4688	0.447	0.4257	0.4744	0.4866	0.4137	0.450	0.4069	0.4498	0.4490	0.3877	0.423	0.3302	0.4446	0.5253	0.4418	0.435	0.2966	0.5047	0.5568	0.3617	0.430	0.2484	0.3546	0.302
21	0.4269	0.4677	0.447	0.4238	0.4738	0.4886	0.4117	0.449	0.4045	0.4452	0.4553	0.3913	0.424	0.3335	0.4468	0.5233	0.4434	0.437	0.2978	0.5042	0.5553	0.3605	0.429	0.2480	0.3532	0.301
22	0.4173	0.4624	0.440	0.4231	0.4725	0.4903	0.4115	0.449	0.3980	0.4463	0.4493	0.3892	0.421	0.3342	0.4472	0.5264	0.4455	0.438	0.2963	0.5045	0.5580	0.3623	0.430	0.2494	0.3528	0.301
23	0.4210	0.4618	0.441	0.4209	0.4699	0.4881	0.4089	0.447	0.3981	0.4462	0.4494	0.3905	0.421	0.3329	0.4473	0.5306	0.4455	0.439	0.2929	0.5072	0.5574	0.3624	0.430	0.2446	0.3530	0.299
24	0.4356	0.4664	0.451	0.4212	0.4680	0.4877	0.4081	0.446	0.4003	0.4445	0.4506	0.3887	0.421	0.3344	0.4477	0.5307	0.4442	0.439	0.3000	0.5055	0.5579	0.3658	0.432	0.2470	0.3527	0.300

Table 10.7 G1 L. monocytogenes growth in MPC broth with various levels of KCl

	KCl																								
	0.00%			0.50%					1.00%					1.50%					2.00%					3.00%		
Time	D2	E2	Average	D3	D4	E3	E4	Average	D5	D6	E5	E6	Average	D7	D8	E7	E8	Average	D9	D10	E9	E10	Average	D11	E11	Average
0	0.1287	0.1190	0.124	0.1172	0.1178	0.1107	0.1154	0.115	0.1044	0.1003	0.0956	0.0994	0.100	0.1034	0.0962	0.1030	0.1101	0.103	0.0939	0.0990	0.0862	0.0959	0.094	0.0967	0.0931	0.095
1	0.1296	0.1279	0.129	0.1255	0.1269	0.1163	0.1223	0.123	0.1128	0.1111	0.1068	0.1094	0.110	0.1088	0.1023	0.1076	0.1143	0.108	0.0948	0.1030	0.0936	0.0992	0.098	0.0984	0.0945	0.096
2	0.1603	0.1551	0.158	0.1599	0.1570	0.1471	0.1544	0.155	0.1414	0.1398	0.1359	0.1387	0.139	0.1318	0.1283	0.1298	0.1432	0.133	0.1126	0.1213	0.1055	0.1174	0.114	0.1148	0.1093	0.112
3	0.2191	0.2126	0.216	0.2209	0.2099	0.2011	0.2095	0.210	0.1879	0.1895	0.1891	0.1861	0.188	0.1760	0.1772	0.1706	0.1882	0.178	0.1506	0.1549	0.1399	0.1503	0.149	0.1419	0.1353	0.139
4	0.3127	0.3126	0.313	0.2919	0.2816	0.2760	0.2756	0.281	0.2479	0.2457	0.2478	0.2466	0.247	0.2287	0.2377	0.2174	0.2383	0.231	0.2020	0.1977	0.1922	0.1990	0.198	0.1875	0.1658	0.177
5	0.4466	0.4464	0.447	0.4120	0.3891	0.3666	0.3691	0.384	0.3522	0.3269	0.3344	0.3281	0.335	0.3081	0.3070	0.2774	0.3124	0.301	0.2509	0.2451	0.2483	0.2450	0.247	0.2189	0.2052	0.212
6	0.6284	0.6237	0.626	0.5455	0.5776	0.5435	0.5909	0.564	0.5226	0.5177	0.4942	0.5245	0.515	0.4545	0.4371	0.4010	0.4794	0.443	0.2977	0.2938	0.2978	0.3041	0.298	0.2658	0.2493	0.258
7	0.5362	0.5441	0.540	0.4920	0.4770	0.5327	0.5296	0.508	0.4412	0.4959	0.4514	0.4979	0.472	0.4652	0.5045	0.5019	0.5029	0.494	0.3616	0.3495	0.3378	0.3519	0.350	0.3163	0.2915	0.304
8	0.5081	0.5189	0.514	0.5084	0.4882	0.5453	0.5189	0.515	0.4518	0.4881	0.4525	0.5134	0.476	0.4484	0.5275	0.4760	0.4847	0.484	0.4244	0.4319	0.4294	0.4355	0.430	0.4016	0.3726	0.387
9	0.5093	0.5096	0.509	0.5173	0.5054	0.5478	0.5263	0.524	0.4505	0.4960	0.4591	0.5221	0.482	0.4456	0.5305	0.4701	0.4891	0.484	0.4792	0.4191	0.4681	0.4639	0.458	0.4321	0.3681	0.400
10	0.5111	0.5141	0.513	0.5214	0.5012	0.5513	0.5296	0.526	0.4532	0.5052	0.4623	0.5269	0.487	0.4446	0.5335	0.4653	0.4862	0.482	0.4936	0.4157	0.4820	0.4802	0.468	0.3709	0.3456	0.358
11	0.5097	0.5125	0.511	0.5239	0.5040	0.5542	0.5262	0.527	0.4509	0.5010	0.4584	0.5234	0.483	0.4434	0.5298	0.4678	0.4833	0.481	0.4982	0.4146	0.4792	0.4816	0.468	0.3694	0.3373	0.353
12	0.5098	0.5138	0.512	0.5228	0.5049	0.5528	0.5293	0.527	0.4497	0.5032	0.4601	0.5234	0.484	0.4429	0.5288	0.4662	0.4826	0.480	0.4999	0.4174	0.4803	0.4824	0.470	0.3683	0.3382	0.353
13	0.5032	0.5123	0.508	0.5214	0.5060	0.5529	0.5301	0.528	0.4480	0.5010	0.4567	0.5227	0.482	0.4399	0.5273	0.4648	0.4791	0.478	0.4967	0.4137	0.4740	0.4776	0.466	0.3712	0.3369	0.354
14	0.5031	0.5058	0.504	0.5221	0.5051	0.5524	0.5333	0.528	0.4480	0.5016	0.4570	0.5221	0.482	0.4408	0.5269	0.4646	0.4792	0.478	0.4970	0.4131	0.4733	0.4771	0.465	0.3682	0.3360	0.352
15	0.5029	0.5029	0.503	0.5212	0.5068	0.5518	0.5278	0.527	0.4467	0.5010	0.4578	0.5223	0.482	0.4401	0.5270	0.4621	0.4797	0.477	0.4942	0.4138	0.4732	0.4756	0.464	0.3668	0.3347	0.351
16	0.5013	0.5023	0.502	0.5211	0.5069	0.5484	0.5272	0.526	0.4465	0.5010	0.4555	0.5210	0.481	0.4426	0.5262	0.4647	0.4794	0.478	0.4960	0.4127	0.4745	0.4790	0.466	0.3714	0.3350	0.353
17	0.4972	0.5044	0.501	0.5214	0.5070	0.5459	0.5315	0.526	0.4463	0.5004	0.4558	0.5205	0.481	0.4447	0.5270	0.4636	0.4800	0.479	0.4992	0.4151	0.4734	0.4804	0.467	0.3734	0.3331	0.353
18	0.4969	0.5015	0.499	0.5189	0.5021	0.5470	0.5269	0.524	0.4454	0.5004	0.4539	0.5189	0.480	0.4427	0.5272	0.4621	0.4795	0.478	0.5000	0.4127	0.4722	0.4785	0.466	0.3719	0.3314	0.352
19	0.4961	0.5012	0.499	0.5179	0.4993	0.5471	0.5287	0.523	0.4439	0.4956	0.4539	0.5169	0.478	0.4431	0.5267	0.4598	0.4794	0.477	0.4979	0.4143	0.4721	0.4777	0.466	0.3722	0.3330	0.353
20	0.4925	0.4960	0.494	0.5188	0.5019	0.5480	0.5286	0.524	0.4423	0.4980	0.4541	0.5134	0.477	0.4439	0.5274	0.4612	0.4805	0.478	0.4994	0.4148	0.4727	0.4795	0.467	0.3699	0.3317	0.351
21	0.4873	0.4911	0.489	0.5168	0.5022	0.5437	0.5285	0.523	0.4409	0.4962	0.4535	0.5120	0.476	0.4435	0.5279	0.4609	0.4785	0.478	0.5008	0.4160	0.4727	0.4823	0.468	0.3663	0.3305	0.348
22	0.4901	0.4889	0.490	0.5150	0.5007	0.5426	0.5254	0.521	0.4408	0.4961	0.4546	0.5112	0.476	0.4473	0.5297	0.4647	0.4776	0.480	0.5020	0.4166	0.4775	0.4821	0.470	0.3666	0.3356	0.351
23	0.4847	0.4883	0.487	0.5143	0.4995	0.5420	0.5252	0.520	0.4397	0.4955	0.4524	0.5116	0.475	0.4497	0.5301	0.4623	0.4776	0.480	0.5041	0.4181	0.4753	0.4821	0.470	0.3678	0.3371	0.352
24	0.4817	0.4837	0.483	0.5144	0.5006	0.5426	0.5240	0.520	0.4399	0.4969	0.4533	0.5144	0.476	0.4502	0.5326	0.4636	0.4810	0.482	0.5078	0.4207	0.4777	0.4892	0.474	0.3717	0.3338	0.353

Table 10.8 G1 L. monocytogenes growth in MPC broth with various levels of $CaCl_2$

	$CaCl_2$																								
	0.00%			0.50%					1.00%					1.50%					2.00%					3.00%		
Time	F2	G2	Average	F3	F4	G3	G4	Average	F5	F6	G5	G6	Average	F7	F8	G7	G8	Average	F9	F10	G9	G10	Average	F11	G11	Average
0	0.1227	0.1196	0.121	1.0269	1.0514	1.1231	1.1043	1.076	1.0084	1.1144	1.0545	1.1271	1.076	0.9168	1.0488	1.0089	1.0257	1.000	0.8651	0.9688	0.9844	0.9544	0.943	0.8465	0.9793	0.913
1	0.1269	0.1309	0.129	0.8324	0.8846	0.8842	0.9230	0.881	0.8550	0.9543	0.9483	0.9401	0.924	0.7883	0.9143	0.8464	0.8742	0.856	0.7592	0.8547	0.8769	0.8266	0.829	0.7449	0.8472	0.796
2	0.1571	0.1668	0.162	0.6699	0.7473	0.7250	0.7763	0.730	0.6826	0.8043	0.8658	0.7904	0.786	0.6280	0.7450	0.6823	0.7325	0.697	0.6292	0.7395	0.7534	0.6851	0.702	0.6406	0.7324	0.687
3	0.2218	0.2258	0.224	0.6006	0.6478	0.6401	0.6802	0.642	0.5768	0.6464	0.7308	0.6374	0.648	0.5226	0.5898	0.5665	0.5836	0.566	0.5128	0.5970	0.6182	0.5428	0.568	0.5398	0.6173	0.579
4	0.3281	0.3349	0.332	0.6144	0.6221	0.6238	0.6576	0.629	0.5475	0.5785	0.5824	0.5663	0.569	0.4908	0.5424	0.5257	0.5189	0.519	0.4673	0.5204	0.5301	0.4913	0.502	0.4799	0.5407	0.510
5	0.4567	0.4868	0.472	0.6418	0.6433	0.6721	0.6971	0.664	0.5655	0.5890	0.5328	0.5665	0.563	0.5038	0.5536	0.5329	0.5117	0.526	0.4570	0.5019	0.5063	0.4798	0.486	0.4689	0.5121	0.491
6	0.6291	0.6750	0.652	0.6697	0.6801	0.7020	0.7394	0.698	0.5935	0.6136	0.5496	0.5985	0.589	0.5294	0.5778	0.5594	0.5349	0.550	0.4735	0.5134	0.5157	0.4965	0.500	0.4712	0.5160	0.494
7	0.5100	0.5331	0.522	0.6893	0.7045	0.7202	0.7567	0.718	0.6134	0.6395	0.5813	0.6238	0.615	0.5466	0.5981	0.5813	0.5592	0.571	0.4907	0.5408	0.5387	0.5175	0.522	0.4759	0.5201	0.498
8	0.5093	0.4908	0.500	0.7012	0.7121	0.7307	0.7627	0.727	0.6251	0.6525	0.5970	0.6398	0.629	0.5632	0.6194	0.5969	0.5800	0.590	0.5144	0.5624	0.5575	0.5329	0.542	0.4886	0.5313	0.510
9	0.5000	0.4902	0.495	0.7045	0.7231	0.7298	0.7678	0.731	0.6353	0.6663	0.6071	0.6460	0.639	0.5785	0.6298	0.6077	0.5920	0.602	0.5322	0.5785	0.5705	0.5497	0.558	0.5011	0.5427	0.522
10	0.5063	0.4951	0.501	0.7071	0.7243	0.7285	0.7649	0.731	0.6382	0.6714	0.6121	0.6513	0.643	0.5826	0.6363	0.6144	0.6013	0.609	0.5420	0.5863	0.5829	0.5609	0.568	0.5093	0.5493	0.529
11	0.5056	0.4947	0.500	0.7032	0.7225	0.7246	0.7639	0.729	0.6383	0.6704	0.6096	0.6514	0.642	0.5852	0.6405	0.6198	0.6075	0.613	0.5506	0.5999	0.5933	0.5675	0.578	0.5174	0.5584	0.538
12	0.5083	0.4879	0.498	0.7011	0.7185	0.7228	0.7618	0.726	0.6393	0.6696	0.6083	0.6511	0.642	0.5858	0.6393	0.6191	0.6077	0.613	0.5522	0.6027	0.5966	0.5726	0.581	0.5245	0.5617	0.543
13	0.5035	0.4917	0.498	0.6967	0.7147	0.7209	0.7591	0.723	0.6364	0.6680	0.6067	0.6479	0.640	0.5847	0.6377	0.6184	0.6084	0.612	0.5510	0.6005	0.5960	0.5721	0.580	0.5279	0.5659	0.547
14	0.5012	0.4877	0.494	0.6960	0.7148	0.7195	0.7582	0.722	0.6365	0.6666	0.6059	0.6473	0.639	0.5842	0.6343	0.6162	0.6046	0.610	0.5504	0.6007	0.5949	0.5708	0.579	0.5306	0.5698	0.550
15	0.5024	0.4849	0.494	0.6941	0.7163	0.7179	0.7574	0.721	0.6360	0.6660	0.6052	0.6464	0.638	0.5834	0.6340	0.6157	0.6033	0.609	0.5507	0.5988	0.5940	0.5690	0.578	0.5303	0.5693	0.550
16	0.4978	0.4856	0.492	0.6914	0.7146	0.7178	0.7558	0.720	0.6359	0.6668	0.6063	0.6467	0.639	0.5838	0.6344	0.6164	0.6054	0.610	0.5499	0.5987	0.5920	0.5678	0.577	0.5283	0.5655	0.547
17	0.4983	0.4836	0.491	0.6903	0.7171	0.7166	0.7562	0.720	0.6363	0.6682	0.6103	0.6485	0.641	0.5851	0.6351	0.6188	0.6059	0.611	0.5524	0.5999	0.5942	0.5697	0.579	0.5270	0.5634	0.545
18	0.4924	0.4799	0.486	0.6905	0.7141	0.7161	0.7560	0.719	0.6375	0.6701	0.6088	0.6495	0.641	0.5854	0.6358	0.6218	0.6071	0.613	0.5542	0.6012	0.5969	0.5718	0.581	0.5260	0.5636	0.545
19	0.4924	0.4768	0.485	0.6895	0.7113	0.7151	0.7558	0.718	0.6397	0.6715	0.6096	0.6513	0.643	0.5890	0.6379	0.6241	0.6099	0.615	0.5566	0.6041	0.5994	0.5742	0.584	0.5256	0.5620	0.544
20	0.4928	0.4678	0.480	0.6887	0.7134	0.7136	0.7545	0.718	0.6399	0.6734	0.6127	0.6530	0.645	0.5891	0.6406	0.6267	0.6123	0.617	0.5579	0.6052	0.6015	0.5773	0.585	0.5272	0.5643	0.546
21	0.4890	0.4740	0.482	0.6895	0.7141	0.7136	0.7541	0.718	0.6407	0.6741	0.6110	0.6537	0.645	0.5912	0.6405	0.6273	0.6136	0.618	0.5600	0.6085	0.6035	0.5798	0.588	0.5273	0.5627	0.545
22	0.4881	0.4653	0.477	0.6891	0.7114	0.7125	0.7543	0.717	0.6429	0.6761	0.6127	0.6544	0.647	0.5929	0.6446	0.6297	0.6157	0.621	0.5637	0.6118	0.6053	0.5812	0.591	0.5293	0.5657	0.548
23	0.4831	0.4626	0.473	0.6894	0.7122	0.7128	0.7534	0.717	0.6431	0.6765	0.6119	0.6547	0.647	0.5938	0.6446	0.6320	0.6176	0.622	0.5642	0.6118	0.6069	0.5843	0.592	0.5299	0.5694	0.550

Table 10.9 G2 L. monocytogenes growth in MPC broth with various levels of NaCl

NaCl																									
	0.00%			0.50%					1.00%					1.50%					2.00%					3.00%		
Time	B2	C2	Average	B3	B4	C3	C4	Average	B5	B6	C5	C6	Average	B7	B8	C7	C8	Average	B9	B10	C9	C10	Average	B11	C11	Average
0	0.109	0.124	0.116	0.121	0.127	0.118	0.130	0.124	0.117	0.104	0.118	0.117	0.114	0.107	0.104	0.097	0.100	0.102	0.102	0.095	0.103	0.102	0.100	0.100	0.110	0.105
1	0.115	0.126	0.120	0.123	0.129	0.119	0.128	0.125	0.119	0.110	0.122	0.125	0.119	0.104	0.105	0.101	0.105	0.104	0.102	0.097	0.111	0.108	0.105	0.099	0.108	0.103
2	0.138	0.147	0.142	0.145	0.153	0.141	0.154	0.148	0.141	0.130	0.147	0.145	0.141	0.123	0.122	0.123	0.125	0.123	0.121	0.116	0.129	0.126	0.123	0.113	0.122	0.117
3	0.179	0.197	0.188	0.189	0.203	0.182	0.202	0.194	0.179	0.167	0.187	0.186	0.180	0.151	0.155	0.158	0.161	0.156	0.156	0.146	0.167	0.164	0.158	0.133	0.152	0.142
4	0.261	0.288	0.274	0.259	0.281	0.244	0.278	0.265	0.235	0.219	0.244	0.241	0.235	0.192	0.193	0.206	0.212	0.201	0.200	0.191	0.217	0.216	0.206	0.163	0.183	0.173
5	0.407	0.438	0.422	0.376	0.411	0.350	0.403	0.385	0.321	0.301	0.330	0.333	0.321	0.260	0.265	0.277	0.282	0.271	0.256	0.245	0.281	0.274	0.264	0.206	0.225	0.216
6	0.579	0.629	0.604	0.566	0.608	0.560	0.616	0.588	0.518	0.479	0.533	0.540	0.517	0.406	0.421	0.455	0.461	0.436	0.340	0.321	0.378	0.370	0.352	0.236	0.264	0.250
7	0.494	0.618	0.556	0.541	0.579	0.621	0.609	0.587	0.594	0.595	0.593	0.556	0.585	0.462	0.473	0.502	0.536	0.493	0.479	0.457	0.519	0.517	0.493	0.276	0.312	0.294
8	0.484	0.611	0.547	0.533	0.574	0.596	0.627	0.583	0.495	0.498	0.531	0.524	0.512	0.392	0.416	0.489	0.525	0.455	0.529	0.521	0.519	0.540	0.527	0.324	0.391	0.357
9	0.472	0.605	0.538	0.526	0.565	0.603	0.639	0.584	0.488	0.487	0.529	0.525	0.507	0.374	0.416	0.492	0.534	0.454	0.541	0.521	0.559	0.588	0.552	0.386	0.450	0.418
10	0.458	0.590	0.524	0.521	0.566	0.600	0.630	0.579	0.478	0.484	0.523	0.520	0.501	0.371	0.413	0.483	0.533	0.450	0.549	0.528	0.563	0.600	0.560	0.395	0.442	0.419
11	0.455	0.575	0.515	0.519	0.559	0.597	0.629	0.576	0.473	0.478	0.521	0.516	0.497	0.373	0.412	0.483	0.533	0.450	0.548	0.530	0.568	0.604	0.562	0.338	0.438	0.388
12	0.450	0.571	0.511	0.513	0.551	0.592	0.620	0.569	0.467	0.476	0.514	0.513	0.492	0.372	0.412	0.479	0.529	0.448	0.548	0.530	0.565	0.599	0.560	0.329	0.437	0.383
13	0.447	0.566	0.507	0.508	0.547	0.586	0.618	0.565	0.464	0.472	0.512	0.509	0.489	0.373	0.411	0.476	0.529	0.447	0.545	0.526	0.560	0.594	0.556	0.327	0.437	0.382
14	0.444	0.561	0.503	0.504	0.541	0.582	0.612	0.560	0.464	0.469	0.510	0.507	0.487	0.372	0.409	0.475	0.525	0.445	0.542	0.523	0.557	0.593	0.554	0.325	0.440	0.382
15	0.439	0.563	0.501	0.499	0.538	0.578	0.607	0.555	0.461	0.467	0.507	0.505	0.485	0.371	0.409	0.474	0.526	0.445	0.539	0.520	0.553	0.590	0.550	0.322	0.439	0.380
16	0.435	0.555	0.495	0.496	0.535	0.575	0.605	0.553	0.459	0.467	0.506	0.504	0.484	0.370	0.408	0.476	0.523	0.444	0.536	0.517	0.550	0.588	0.548	0.319	0.437	0.378
17	0.434	0.555	0.494	0.494	0.532	0.573	0.602	0.550	0.459	0.463	0.505	0.503	0.483	0.371	0.408	0.473	0.522	0.443	0.534	0.516	0.548	0.585	0.545	0.319	0.433	0.376
18	0.427	0.551	0.489	0.491	0.529	0.570	0.599	0.547	0.457	0.461	0.505	0.501	0.481	0.371	0.408	0.473	0.522	0.443	0.531	0.513	0.545	0.583	0.543	0.316	0.431	0.373
19	0.427	0.546	0.486	0.490	0.527	0.566	0.597	0.545	0.456	0.458	0.502	0.500	0.479	0.371	0.406	0.473	0.521	0.443	0.530	0.512	0.544	0.582	0.542	0.312	0.427	0.369
20	0.422	0.546	0.484	0.487	0.525	0.566	0.595	0.543	0.454	0.457	0.500	0.499	0.477	0.372	0.408	0.471	0.518	0.442	0.530	0.512	0.541	0.581	0.541	0.312	0.426	0.369
21	0.417	0.545	0.481	0.487	0.524	0.564	0.594	0.542	0.453	0.456	0.499	0.498	0.476	0.373	0.406	0.471	0.519	0.442	0.530	0.512	0.540	0.581	0.541	0.312	0.423	0.367
22	0.411	0.537	0.474	0.484	0.523	0.561	0.592	0.540	0.451	0.455	0.497	0.496	0.475	0.373	0.406	0.471	0.519	0.442	0.529	0.511	0.540	0.579	0.540	0.313	0.424	0.369
23	0.411	0.537	0.474	0.483	0.521	0.560	0.591	0.538	0.457	0.454	0.496	0.496	0.476	0.375	0.405	0.470	0.516	0.442	0.528	0.510	0.538	0.578	0.539	0.311	0.423	0.367
24	0.403	0.528	0.465	0.482	0.519	0.558	0.589	0.537	0.450	0.453	0.495	0.495	0.473	0.375	0.405	0.469	0.516	0.441	0.529	0.511	0.537	0.578	0.539	0.313	0.424	0.369

Table 10.10 G2 L. monocytogenes growth in MPC broth with various levels of KCl

KCl																										
	0.00%			0.50%					1.00%					1.50%					2.00%					3.00%		
Time	D2	E2	AVG	D3	D4	E3	E4	AVG	D5	D6	E5	E6	AVG	D7	D8	E7	E8	AVG	D9	D10	E9	E10	AVG	D11	E11	AVG
0	0.128	0.118	0.123	0.120	0.111	0.117	0.115	0.116	0.106	0.101	0.098	0.100	0.101	0.099	0.097	0.095	0.097	0.097	0.089	0.105	0.098	0.089	0.095	0.105	0.108	0.107
1	0.125	0.125	0.125	0.123	0.115	0.122	0.119	0.120	0.108	0.107	0.103	0.102	0.105	0.104	0.102	0.099	0.100	0.101	0.092	0.104	0.100	0.091	0.097	0.105	0.107	0.106
2	0.147	0.152	0.149	0.147	0.139	0.146	0.144	0.144	0.134	0.132	0.127	0.126	0.130	0.124	0.122	0.119	0.120	0.121	0.105	0.117	0.116	0.105	0.111	0.118	0.122	0.120
3	0.196	0.196	0.196	0.197	0.187	0.193	0.193	0.192	0.178	0.177	0.172	0.171	0.175	0.163	0.160	0.156	0.160	0.160	0.133	0.150	0.148	0.131	0.141	0.149	0.155	0.152
4	0.282	0.286	0.284	0.268	0.253	0.264	0.260	0.261	0.239	0.236	0.226	0.228	0.233	0.211	0.211	0.204	0.208	0.209	0.180	0.199	0.195	0.176	0.187	0.191	0.201	0.196
5	0.456	0.458	0.457	0.426	0.379	0.404	0.396	0.401	0.359	0.362	0.326	0.331	0.345	0.283	0.281	0.271	0.279	0.279	0.235	0.245	0.235	0.230	0.236	0.236	0.244	0.240
6	0.668	0.678	0.673	0.581	0.628	0.612	0.648	0.617	0.595	0.598	0.589	0.596	0.594	0.429	0.425	0.402	0.418	0.419	0.292	0.313	0.285	0.287	0.294	0.291	0.298	0.294
7	0.682	0.655	0.669	0.562	0.606	0.595	0.631	0.599	0.588	0.586	0.565	0.573	0.578	0.552	0.553	0.532	0.547	0.546	0.352	0.415	0.365	0.352	0.371	0.380	0.387	0.384
8	0.677	0.646	0.661	0.575	0.617	0.607	0.642	0.610	0.610	0.598	0.591	0.592	0.598	0.535	0.536	0.536	0.540	0.537	0.465	0.448	0.464	0.464	0.460	0.438	0.463	0.450
9	0.673	0.651	0.662	0.577	0.622	0.612	0.649	0.615	0.613	0.605	0.598	0.596	0.603	0.550	0.561	0.546	0.548	0.551	0.510	0.456	0.483	0.499	0.487	0.476	0.487	0.481
10	0.664	0.630	0.647	0.578	0.612	0.609	0.639	0.610	0.610	0.597	0.587	0.588	0.595	0.546	0.562	0.548	0.551	0.552	0.512	0.464	0.477	0.490	0.485	0.483	0.509	0.496
11	0.653	0.621	0.637	0.575	0.607	0.605	0.632	0.605	0.608	0.592	0.584	0.585	0.592	0.549	0.564	0.550	0.549	0.553	0.514	0.467	0.487	0.493	0.490	0.480	0.515	0.497
12	0.648	0.614	0.631	0.572	0.602	0.603	0.625	0.601	0.602	0.588	0.576	0.579	0.586	0.543	0.556	0.546	0.545	0.548	0.514	0.468	0.487	0.494	0.491	0.482	0.516	0.499
13	0.643	0.606	0.625	0.570	0.598	0.598	0.620	0.596	0.597	0.583	0.572	0.574	0.582	0.540	0.554	0.545	0.542	0.545	0.512	0.466	0.483	0.490	0.487	0.480	0.515	0.498
14	0.638	0.603	0.620	0.564	0.593	0.592	0.613	0.590	0.592	0.579	0.567	0.568	0.577	0.533	0.549	0.540	0.537	0.540	0.508	0.463	0.481	0.486	0.484	0.476	0.510	0.493
15	0.633	0.598	0.616	0.562	0.587	0.588	0.609	0.586	0.587	0.575	0.560	0.564	0.571	0.529	0.543	0.536	0.533	0.535	0.505	0.460	0.478	0.484	0.482	0.474	0.507	0.490
16	0.630	0.593	0.612	0.560	0.584	0.586	0.606	0.584	0.586	0.573	0.559	0.561	0.570	0.525	0.540	0.533	0.530	0.532	0.502	0.460	0.476	0.481	0.480	0.475	0.505	0.490
17	0.628	0.590	0.609	0.559	0.582	0.584	0.603	0.582	0.584	0.571	0.556	0.557	0.567	0.524	0.538	0.530	0.527	0.530	0.500	0.458	0.473	0.477	0.477	0.471	0.503	0.487
18	0.625	0.588	0.607	0.556	0.579	0.580	0.600	0.579	0.580	0.568	0.552	0.556	0.564	0.520	0.535	0.528	0.526	0.527	0.496	0.456	0.470	0.475	0.474	0.470	0.500	0.485
19	0.623	0.587	0.605	0.556	0.576	0.580	0.597	0.577	0.579	0.567	0.550	0.552	0.562	0.519	0.533	0.527	0.522	0.525	0.495	0.454	0.468	0.474	0.473	0.468	0.497	0.482
20	0.620	0.582	0.601	0.553	0.574	0.578	0.593	0.574	0.576	0.563	0.548	0.551	0.559	0.516	0.530	0.525	0.520	0.523	0.494	0.453	0.466	0.471	0.471	0.466	0.498	0.482
21	0.617	0.581	0.599	0.553	0.573	0.575	0.593	0.573	0.576	0.563	0.546	0.549	0.559	0.515	0.529	0.523	0.520	0.522	0.493	0.452	0.468	0.470	0.471	0.465	0.496	0.480
22	0.615	0.578	0.597	0.552	0.572	0.574	0.590	0.572	0.574	0.561	0.544	0.547	0.557	0.514	0.527	0.522	0.518	0.520	0.492	0.450	0.465	0.469	0.469	0.464	0.494	0.479
23	0.613	0.577	0.595	0.550	0.569	0.572	0.588	0.570	0.572	0.559	0.542	0.545	0.555	0.512	0.526	0.520	0.516	0.519	0.490	0.450	0.464	0.470	0.469	0.463	0.493	0.478
24	0.611	0.573	0.592	0.548	0.567	0.571	0.586	0.568	0.570	0.558	0.540	0.544	0.553	0.510	0.524	0.519	0.515	0.517	0.489	0.450	0.464	0.469	0.468	0.461	0.492	0.476

Table 10.11 G2 L. monocytogenes growth in MPC broth with various levels of $CaCl_2$

	$CaCl_2$																								
	0.00%			0.50%					1.00%					1.50%					2.00%					3.00%		
Time	F2	G2	AVG	F3	F4	G3	G4	AVG	F5	F6	G5	G6	AVG	F7	F8	G7	G8	AVG	F9	F10	G9	G10	AVG	F11	G11	AVG
0	0.121	0.116	0.118	1.155	1.134	1.144	1.151	1.146	1.097	1.101	1.065	1.110	1.093	1.081	0.966	1.004	0.990	1.010	0.972	0.996	0.906	0.948	0.956	0.963	0.993	0.978
1	0.126	0.125	0.126	0.984	0.988	0.968	0.986	0.981	0.970	0.993	0.922	0.953	0.959	0.976	0.868	0.886	0.862	0.898	0.884	0.898	0.806	0.839	0.857	0.864	0.853	0.859
2	0.153	0.155	0.154	0.856	0.854	0.853	0.861	0.856	0.860	0.894	0.813	0.861	0.857	0.893	0.783	0.806	0.751	0.808	0.826	0.846	0.722	0.745	0.785	0.799	0.786	0.792
3	0.206	0.207	0.207	0.741	0.731	0.736	0.743	0.738	0.715	0.752	0.676	0.735	0.720	0.753	0.646	0.698	0.632	0.682	0.704	0.740	0.607	0.630	0.670	0.697	0.693	0.695
4	0.290	0.300	0.295	0.678	0.674	0.669	0.680	0.675	0.633	0.644	0.603	0.624	0.626	0.629	0.558	0.591	0.568	0.587	0.585	0.604	0.520	0.550	0.565	0.605	0.601	0.603
5	0.473	0.481	0.477	0.715	0.713	0.704	0.719	0.713	0.625	0.622	0.607	0.602	0.614	0.598	0.541	0.565	0.562	0.567	0.546	0.545	0.500	0.534	0.531	0.549	0.543	0.546
6	0.697	0.688	0.693	0.760	0.772	0.758	0.769	0.765	0.675	0.670	0.649	0.638	0.658	0.630	0.579	0.585	0.595	0.597	0.549	0.542	0.506	0.543	0.535	0.542	0.530	0.536
7	0.700	0.568	0.634	0.801	0.801	0.793	0.802	0.799	0.705	0.704	0.690	0.685	0.696	0.668	0.615	0.630	0.633	0.636	0.577	0.569	0.538	0.576	0.565	0.552	0.540	0.546
8	0.691	0.545	0.618	0.819	0.820	0.809	0.822	0.817	0.744	0.739	0.719	0.714	0.729	0.702	0.650	0.665	0.667	0.671	0.613	0.603	0.570	0.605	0.598	0.570	0.554	0.562
9	0.689	0.534	0.611	0.827	0.826	0.813	0.825	0.823	0.763	0.758	0.730	0.732	0.746	0.728	0.674	0.688	0.687	0.694	0.640	0.634	0.593	0.632	0.625	0.589	0.573	0.581
10	0.674	0.516	0.595	0.819	0.816	0.803	0.817	0.814	0.761	0.755	0.726	0.725	0.742	0.741	0.684	0.690	0.689	0.701	0.658	0.653	0.609	0.647	0.642	0.606	0.586	0.596
11	0.664	0.504	0.584	0.812	0.811	0.798	0.812	0.808	0.756	0.752	0.719	0.719	0.737	0.737	0.678	0.683	0.685	0.696	0.671	0.662	0.612	0.655	0.650	0.619	0.597	0.608
12	0.658	0.502	0.580	0.805	0.808	0.795	0.806	0.803	0.751	0.745	0.717	0.714	0.732	0.732	0.672	0.680	0.682	0.692	0.664	0.657	0.607	0.648	0.644	0.630	0.606	0.618
13	0.652	0.493	0.572	0.801	0.804	0.791	0.802	0.799	0.749	0.743	0.715	0.712	0.730	0.731	0.672	0.677	0.679	0.689	0.661	0.654	0.602	0.643	0.640	0.626	0.602	0.614
14	0.648	0.489	0.569	0.798	0.801	0.786	0.797	0.795	0.746	0.742	0.711	0.708	0.727	0.727	0.669	0.671	0.673	0.685	0.659	0.650	0.600	0.639	0.637	0.619	0.595	0.607
15	0.645	0.484	0.564	0.793	0.794	0.783	0.793	0.791	0.739	0.737	0.705	0.703	0.721	0.719	0.663	0.662	0.667	0.678	0.653	0.642	0.593	0.631	0.630	0.616	0.591	0.604
16	0.643	0.482	0.562	0.790	0.793	0.779	0.791	0.788	0.739	0.735	0.701	0.700	0.719	0.716	0.661	0.660	0.663	0.675	0.649	0.639	0.588	0.626	0.626	0.613	0.586	0.599
17	0.638	0.479	0.558	0.787	0.791	0.778	0.787	0.786	0.733	0.732	0.699	0.695	0.715	0.713	0.656	0.656	0.660	0.671	0.644	0.636	0.583	0.622	0.621	0.607	0.580	0.593
18	0.637	0.475	0.556	0.783	0.789	0.775	0.783	0.783	0.731	0.728	0.694	0.691	0.711	0.709	0.653	0.651	0.656	0.667	0.640	0.631	0.579	0.617	0.617	0.603	0.575	0.589
19	0.633	0.479	0.556	0.782	0.787	0.773	0.782	0.781	0.728	0.726	0.693	0.690	0.709	0.704	0.651	0.649	0.654	0.665	0.638	0.628	0.578	0.615	0.614	0.598	0.572	0.585
20	0.630	0.470	0.550	0.778	0.783	0.771	0.778	0.777	0.727	0.721	0.690	0.688	0.706	0.702	0.647	0.646	0.650	0.661	0.633	0.623	0.573	0.612	0.610	0.596	0.568	0.582
21	0.628	0.468	0.548	0.776	0.781	0.769	0.776	0.776	0.726	0.722	0.688	0.684	0.705	0.701	0.646	0.642	0.647	0.659	0.632	0.622	0.572	0.608	0.608	0.592	0.564	0.578
22	0.625	0.475	0.550	0.774	0.780	0.767	0.774	0.774	0.723	0.720	0.686	0.683	0.703	0.697	0.643	0.642	0.645	0.657	0.629	0.619	0.569	0.606	0.606	0.590	0.562	0.576
23	0.622	0.463	0.543	0.772	0.777	0.765	0.772	0.772	0.722	0.717	0.684	0.683	0.701	0.695	0.642	0.638	0.644	0.655	0.626	0.616	0.567	0.603	0.603	0.588	0.558	0.573
24	0.619	0.460	0.540	0.769	0.774	0.764	0.770	0.769	0.719	0.716	0.681	0.679	0.699	0.692	0.638	0.634	0.641	0.651	0.624	0.615	0.564	0.600	0.601	0.584	0.556	0.570

Table 10.12 Effect of various salt levels on G1 Listeria monocytogenes growth rates in liquid phase with 0.06% Potassium Sorbate

Time	NaCl %					KCl %					$CaCl_2$ %				
	0.000	0.500	1.000	1.500	2.000	0.000	0.500	1.000	1.500	2.000	0.000	0.500	1.000	1.500	2.000
0.000	0.033	0.030	0.030	0.033	0.037	0.033	0.032	0.029	0.032	0.030	0.033	0.031	0.031	0.032	0.030
1.000	0.033	0.030	0.031	0.032	0.036	0.033	0.033	0.029	0.035	0.029	0.033	0.032	0.032	0.031	0.030
2.000	0.033	0.029	0.031	0.032	0.036	0.033	0.033	0.028	0.036	0.028	0.033	0.031	0.032	0.032	0.031
3.000	0.033	0.029	0.030	0.032	0.036	0.033	0.033	0.029	0.035	0.029	0.033	0.031	0.032	0.032	0.031
4.000	0.032	0.029	0.030	0.032	0.036	0.032	0.033	0.029	0.035	0.030	0.032	0.031	0.032	0.032	0.031
5.000	0.033	0.030	0.030	0.032	0.035	0.033	0.032	0.029	0.035	0.029	0.033	0.031	0.032	0.032	0.031
6.000	0.033	0.030	0.030	0.032	0.035	0.033	0.033	0.029	0.035	0.029	0.033	0.032	0.032	0.032	0.031
7.000	0.033	0.030	0.030	0.032	0.035	0.033	0.033	0.029	0.036	0.029	0.033	0.032	0.032	0.032	0.032
8.000	0.033	0.029	0.030	0.032	0.033	0.033	0.032	0.029	0.034	0.029	0.033	0.031	0.032	0.032	0.031
9.000	0.033	0.030	0.031	0.032	0.033	0.033	0.032	0.029	0.035	0.030	0.033	0.032	0.033	0.033	0.031
10.000	0.033	0.030	0.029	0.032	0.034	0.033	0.034	0.029	0.035	0.029	0.033	0.032	0.032	0.033	0.032
11.000	0.033	0.029	0.030	0.032	0.033	0.033	0.034	0.029	0.036	0.029	0.033	0.032	0.032	0.033	0.032
12.000	0.033	0.030	0.030	0.033	0.034	0.033	0.034	0.029	0.036	0.030	0.033	0.032	0.033	0.033	0.032
13.000	0.033	0.030	0.030	0.033	0.034	0.033	0.034	0.029	0.035	0.031	0.033	0.033	0.033	0.033	0.032
14.000	0.033	0.030	0.030	0.033	0.034	0.033	0.033	0.029	0.036	0.031	0.033	0.032	0.033	0.033	0.032
15.000	0.033	0.030	0.031	0.033	0.034	0.033	0.034	0.029	0.035	0.031	0.033	0.032	0.033	0.033	0.032
16.000	0.033	0.031	0.031	0.032	0.033	0.033	0.034	0.030	0.036	0.031	0.033	0.032	0.033	0.033	0.032
17.000	0.033	0.030	0.030	0.033	0.034	0.033	0.033	0.030	0.035	0.031	0.033	0.032	0.032	0.033	0.033
18.000	0.034	0.030	0.031	0.032	0.034	0.034	0.034	0.030	0.035	0.032	0.034	0.033	0.033	0.034	0.032
19.000	0.034	0.030	0.030	0.033	0.034	0.034	0.034	0.030	0.035	0.032	0.034	0.032	0.033	0.034	0.033
20.000	0.033	0.030	0.030	0.033	0.033	0.033	0.034	0.030	0.035	0.031	0.033	0.032	0.032	0.033	0.032
21.000	0.033	0.031	0.032	0.032	0.034	0.033	0.034	0.030	0.034	0.032	0.033	0.033	0.033	0.034	0.033
22.000	0.033	0.030	0.030	0.032	0.033	0.033	0.033	0.029	0.034	0.031	0.033	0.033	0.032	0.034	0.032
23.000	0.034	0.030	0.031	0.032	0.034	0.034	0.034	0.029	0.034	0.031	0.034	0.033	0.033	0.034	0.032
24.000	0.033	0.030	0.031	0.032	0.034	0.033	0.034	0.030	0.034	0.031	0.033	0.032	0.032	0.034	0.032

Table 10.13 Effect of various salt levels on G2 Listeria monocytogenes growth rates in liquid phase with 0.06% Potassium Sorbate

G2															
Time	NaCl %					KCl %					$CaCl_2$ %				
	0.000	0.500	1.000	1.500	2.000	0.000	0.500	1.000	1.500	2.000	0.000	0.500	1.000	1.500	2.000
0.000	0.033	0.031	0.031	0.030	0.029	0.031	0.029	0.030	0.029	0.030	0.033	0.030	0.031	0.031	0.030
1.000	0.033	0.032	0.032	0.030	0.029	0.033	0.030	0.030	0.029	0.030	0.032	0.030	0.031	0.032	0.031
2.000	0.033	0.031	0.032	0.031	0.029	0.032	0.030	0.030	0.029	0.030	0.033	0.030	0.031	0.032	0.031
3.000	0.033	0.032	0.031	0.031	0.030	0.032	0.029	0.030	0.029	0.029	0.032	0.030	0.032	0.032	0.031
4.000	0.032	0.031	0.032	0.031	0.030	0.032	0.029	0.030	0.029	0.029	0.032	0.031	0.031	0.032	0.031
5.000	0.033	0.031	0.031	0.031	0.030	0.032	0.030	0.030	0.029	0.030	0.033	0.031	0.031	0.032	0.031
6.000	0.033	0.031	0.031	0.031	0.029	0.032	0.030	0.030	0.029	0.030	0.033	0.031	0.032	0.032	0.032
7.000	0.033	0.032	0.032	0.032	0.029	0.032	0.030	0.030	0.029	0.030	0.033	0.031	0.032	0.032	0.032
8.000	0.033	0.032	0.032	0.031	0.029	0.032	0.030	0.030	0.029	0.029	0.032	0.031	0.031	0.032	0.032
9.000	0.033	0.032	0.031	0.032	0.029	0.032	0.030	0.030	0.029	0.030	0.033	0.031	0.032	0.032	0.032
10.000	0.033	0.032	0.032	0.031	0.030	0.032	0.030	0.030	0.030	0.030	0.032	0.031	0.032	0.033	0.033
11.000	0.033	0.032	0.032	0.031	0.030	0.032	0.030	0.030	0.030	0.030	0.033	0.031	0.033	0.034	0.033
12.000	0.033	0.031	0.032	0.032	0.030	0.032	0.030	0.030	0.029	0.030	0.032	0.031	0.032	0.033	0.033
13.000	0.033	0.032	0.032	0.032	0.029	0.032	0.031	0.030	0.029	0.030	0.032	0.031	0.033	0.033	0.033
14.000	0.033	0.032	0.031	0.032	0.030	0.032	0.030	0.030	0.029	0.030	0.033	0.031	0.033	0.034	0.033
15.000	0.033	0.032	0.032	0.032	0.030	0.033	0.031	0.030	0.030	0.030	0.033	0.032	0.033	0.034	0.033
16.000	0.033	0.032	0.032	0.032	0.030	0.032	0.031	0.030	0.030	0.030	0.033	0.031	0.033	0.034	0.033
17.000	0.033	0.032	0.032	0.032	0.030	0.032	0.030	0.030	0.029	0.031	0.033	0.031	0.032	0.034	0.033
18.000	0.034	0.032	0.032	0.032	0.030	0.033	0.031	0.030	0.029	0.030	0.033	0.031	0.033	0.034	0.033
19.000	0.034	0.032	0.032	0.032	0.030	0.032	0.030	0.030	0.030	0.030	0.034	0.032	0.033	0.033	0.033
20.000	0.033	0.032	0.032	0.032	0.030	0.033	0.030	0.030	0.029	0.030	0.033	0.032	0.033	0.034	0.033
21.000	0.033	0.032	0.032	0.032	0.031	0.032	0.030	0.031	0.029	0.030	0.033	0.032	0.033	0.035	0.033
22.000	0.033	0.032	0.032	0.032	0.030	0.032	0.030	0.030	0.029	0.031	0.033	0.032	0.032	0.034	0.033
23.000	0.034	0.033	0.032	0.032	0.030	0.032	0.031	0.030	0.030	0.030	0.033	0.032	0.032	0.034	0.033
24.000	0.033	0.032	0.032	0.032	0.031	0.032	0.031	0.030	0.029	0.031	0.033	0.032	0.033	0.034	0.033

Table 10.14 Effect of various salt levels on G1 Listeria monocytogenes growth rates in liquid phase with no Potassium Sorbate

G1															
	NaCl %					KCl %					$CaCl_2$ %				
Time	0.0	0.5	1.0	1.5	2.0	0.0	0.5	1.0	1.5	2.0	0.0	0.5	1.0	1.5	2.0
0	0.031	0.0322	0.0301	0.0330	0.0370	0.031	0.0314	0.0310	0.0315	0.0306	0.031	0.0307	0.0309	0.0309	0.0302
1	0.031	0.0318	0.0307	0.0318	0.0359	0.031	0.0312	0.0310	0.0322	0.0305	0.031	0.0310	0.0312	0.0309	0.0305
2	0.031	0.0328	0.0307	0.0318	0.0361	0.031	0.0319	0.0314	0.0330	0.0306	0.031	0.0312	0.0316	0.0313	0.0311
3	0.034	0.0334	0.0315	0.0320	0.0360	0.034	0.0339	0.0341	0.0344	0.0315	0.034	0.0334	0.0329	0.0331	0.0318
4	0.036	0.0356	0.0333	0.0345	0.0339	0.036	0.0356	0.0357	0.0355	0.0326	0.036	0.0342	0.0333	0.0338	0.0323
5	0.037	0.0366	0.0412	0.0389	0.0301	0.037	0.0366	0.0367	0.0361	0.0326	0.037	0.0350	0.0341	0.0345	0.0329
6	0.036	0.0380	0.0425	0.0394	0.0310	0.036	0.0372	0.0367	0.0364	0.0325	0.036	0.0349	0.0340	0.0344	0.0326
7	0.041	0.0382	0.0455	0.0433	0.0344	0.041	0.0397	0.0404	0.0386	0.0338	0.041	0.0381	0.0361	0.0369	0.0345
8	0.041	0.0391	0.0511	0.0461	0.0386	0.041	0.0401	0.0406	0.0384	0.0336	0.041	0.0380	0.0361	0.0368	0.0338
9	0.052	0.0412	0.0556	0.0539	0.0448	0.052	0.0467	0.0495	0.0438	0.0367	0.052	0.0456	0.0413	0.0429	0.0371
10	0.062	0.0420	0.0601	0.0609	0.0505	0.062	0.0518	0.0567	0.0479	0.0384	0.062	0.0518	0.0453	0.0478	0.0397
11	0.065	0.0467	0.0611	0.0629	0.0520	0.065	0.0557	0.0602	0.0505	0.0398	0.065	0.0537	0.0466	0.0496	0.0406
12	0.072	0.0482	0.0622	0.0669	0.0546	0.072	0.0599	0.0658	0.0538	0.0417	0.072	0.0585	0.0498	0.0533	0.0425
13	0.073	0.0501	0.0605	0.0669	0.0537	0.073	0.0617	0.0675	0.0549	0.0430	0.073	0.0598	0.0507	0.0542	0.0430
14	0.079	0.0522	0.0615	0.0702	0.0559	0.079	0.0656	0.0722	0.0578	0.0444	0.079	0.0633	0.0531	0.0571	0.0446
15	0.096	0.0525	0.0622	0.0789	0.0606	0.096	0.0741	0.0849	0.0648	0.0477	0.096	0.0746	0.0608	0.0660	0.0491
16	0.132	0.0536	0.0635	0.0978	0.0706	0.132	0.0928	0.1124	0.0804	0.0555	0.132	0.0988	0.0767	0.0852	0.0587
17	0.156	0.0555	0.0661	0.1111	0.0786	0.156	0.1058	0.1309	0.0906	0.0609	0.156	0.1147	0.0873	0.0977	0.0652
18	0.168	0.0602	0.0701	0.1190	0.0845	0.168	0.1140	0.1409	0.0966	0.0641	0.168	0.1228	0.0927	0.1043	0.0683
19	0.178	0.0655	0.0703	0.1243	0.0873	0.178	0.1219	0.1501	0.1022	0.0671	0.178	0.1296	0.0973	0.1099	0.0712
20	0.180	0.0682	0.0714	0.1257	0.0886	0.180	0.1241	0.1521	0.1036	0.0673	0.180	0.1308	0.0980	0.1105	0.0713
21	0.185	0.0721	0.0758	0.1304	0.0931	0.185	0.1286	0.1568	0.1065	0.0690	0.185	0.1342	0.1004	0.1133	0.0731
22	0.199	0.0786	0.0800	0.1394	0.0997	0.199	0.1387	0.1687	0.1138	0.0725	0.199	0.1433	0.1063	0.1206	0.0765
23	0.234	0.0821	0.0812	0.1576	0.1094	0.234	0.1581	0.1960	0.1294	0.0803	0.234	0.1668	0.1221	0.1392	0.0857
24	0.279	0.0881	0.0820	0.1805	0.1213	0.279	0.1836	0.2313	0.1496	0.0903	0.279	0.1967	0.1419	0.1629	0.0975

Table 10.15 Effect of various salt levels on G2 Listeria monocytogenes growth rates in liquid phase with no Potassium Sorbate

G2															
	NaCl %					KCl %					$CaCl_2$ %				
Time	0.0	0.5	1.0	1.5	2.0	0.0	0.5	1.0	1.5	2.0	0.0	0.5	1.0	1.5	2.0
0	0.0328	0.0311	0.0313	0.0314	0.0331	0.0328	0.0310	0.0315	0.0307	0.0298	0.0328	0.0316	0.0317	0.0311	0.0300
1	0.0330	0.0311	0.0314	0.0314	0.0321	0.0330	0.0312	0.0314	0.0306	0.0302	0.0330	0.0314	0.0319	0.0318	0.0303
2	0.0332	0.0315	0.0318	0.0316	0.0323	0.0332	0.0315	0.0316	0.0312	0.0300	0.0332	0.0319	0.0321	0.0321	0.0306
3	0.0367	0.0334	0.0327	0.0321	0.0322	0.0367	0.0336	0.0338	0.0323	0.0304	0.0367	0.0336	0.0338	0.0329	0.0313
4	0.0380	0.0346	0.0339	0.0336	0.0324	0.0380	0.0349	0.0346	0.0329	0.0312	0.0380	0.0346	0.0345	0.0333	0.0314
5	0.0390	0.0352	0.0362	0.0375	0.0328	0.0390	0.0357	0.0355	0.0334	0.0310	0.0390	0.0351	0.0352	0.0336	0.0317
6	0.0387	0.0354	0.0369	0.0380	0.0331	0.0387	0.0357	0.0353	0.0335	0.0312	0.0387	0.0352	0.0353	0.0341	0.0318
7	0.0436	0.0380	0.0385	0.0403	0.0355	0.0436	0.0386	0.0384	0.0352	0.0317	0.0436	0.0375	0.0374	0.0353	0.0327
8	0.0435	0.0383	0.0403	0.0427	0.0379	0.0435	0.0387	0.0385	0.0353	0.0314	0.0435	0.0375	0.0373	0.0352	0.0325
9	0.0547	0.0444	0.0433	0.0465	0.0424	0.0547	0.0460	0.0451	0.0392	0.0330	0.0547	0.0430	0.0430	0.0385	0.0341
10	0.0642	0.0494	0.0461	0.0499	0.0469	0.0642	0.0520	0.0508	0.0426	0.0341	0.0642	0.0477	0.0476	0.0411	0.0358
11	0.0673	0.0518	0.0481	0.0513	0.0481	0.0673	0.0546	0.0532	0.0443	0.0349	0.0673	0.0496	0.0493	0.0423	0.0364
12	0.0743	0.0558	0.0497	0.0532	0.0504	0.0743	0.0593	0.0575	0.0466	0.0360	0.0743	0.0528	0.0524	0.0438	0.0378
13	0.0761	0.0572	0.0501	0.0528	0.0497	0.0761	0.0607	0.0588	0.0477	0.0363	0.0761	0.0537	0.0533	0.0445	0.0373
14	0.0817	0.0605	0.0516	0.0542	0.0518	0.0817	0.0644	0.0621	0.0496	0.0371	0.0817	0.0565	0.0560	0.0461	0.0382
15	0.0986	0.0691	0.0540	0.0574	0.0562	0.0986	0.0751	0.0722	0.0556	0.0390	0.0986	0.0652	0.0644	0.0505	0.0408
16	0.1354	0.0877	0.0594	0.0638	0.0660	0.1354	0.0980	0.0941	0.0679	0.0425	0.1354	0.0834	0.0822	0.0603	0.0455
17	0.1596	0.1002	0.0635	0.0687	0.0731	0.1596	0.1131	0.1085	0.0761	0.0455	0.1596	0.0952	0.0938	0.0666	0.0487
18	0.1715	0.1073	0.0676	0.0728	0.0777	0.1715	0.1212	0.1163	0.0806	0.0471	0.1715	0.1015	0.0997	0.0697	0.0505
19	0.1821	0.1139	0.0706	0.0749	0.0805	0.1821	0.1283	0.1230	0.0850	0.0485	0.1821	0.1070	0.1050	0.0724	0.0517
20	0.1838	0.1153	0.0721	0.0759	0.0812	0.1838	0.1296	0.1241	0.0857	0.0486	0.1838	0.1078	0.1056	0.0728	0.0515
21	0.1889	0.1188	0.0749	0.0787	0.0844	0.1889	0.1332	0.1275	0.0878	0.0497	0.1889	0.1105	0.1082	0.0745	0.0527
22	0.2027	0.1274	0.0798	0.0834	0.0894	0.2027	0.1426	0.1362	0.0930	0.0514	0.2027	0.1175	0.1148	0.0780	0.0545
23	0.2383	0.1462	0.0855	0.0897	0.0989	0.2383	0.1654	0.1577	0.1054	0.0552	0.2383	0.1352	0.1320	0.0873	0.0591
24	0.2838	0.1701	0.0934	0.0975	0.1104	0.2838	0.1944	0.1851	0.1214	0.0606	0.2838	0.1580	0.1542	0.0991	0.0653

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